THE IMPORTANCE OF THE ANGIOTENSIN TYPE-1 RECEPTOR IN THE VASCULAR RESPONSE TO INJURY A STUDY WITH AUTOIMMUNIZATION AND ANTISENSE

By

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This dissertation is dedicated to my wife, Jane for all the love, care, support and encouragement she has given me through the years

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ABBREVIATIONS

ACE	Angiotensin Converting Enzyme
ACSF	Artificial Cerebelspinal Fluid
Ang II	Angiotensin II
Ao	Angiotensinogen
AP-1	Activator protein 1
AS-ODN	Antisense Oligodeoxynucleotides
AT_1	Angiotensin type-1 receptor
AT_2	Angiotensin type-2 receptor
AVP	Arginin Vasopressin
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bFGF	basic fibroblast growth factor
BSA	Bovine Serum Albumin
CMV	Cytomegalovirus
CNS	Central Nervous System
DEN	Starburst® dendrimers
ELISA	Enzyme Linked Immunosorbent Assay
EtOH	Ethanol
FITC	Fluorescein Isothiocyanate

G-protein	Guanine Nucleotide Binding Protein
MAP kinase	Mitogen associated protein Kinase
MAP	Multiple Antigenic Peptides
MECATOR	Multicenter European Research trial with Cilazapril after Angioplasty to prevent Transluminal Coronary Obstruction and Restenosis
NO	Nitric Oxide
PBS	Phosphate buffered Saline
PKC	protein kinase C
RAS	Renin angiotensin system
SC-ODN	Scrambled Oligodeoxynucleotides
VSMC	Vascular Smooth Muscle Cell

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Arterial injury induces VSMC proliferation and migration. This leads to neointimal growth and reduction in lumenal diameter. The local RAS has been suggested to promote this process mediated by AT_1 receptor. To test the role of angiotensin II in a rat common carotid artery model of restenosis, we utilized autoimmunization and antisense inhibition strategies.

In the autoimmunization study, SD rats were immunized with a synthetic peptide corresponding to amino acid sequence 14-23 of the N-terminal the AT_1 receptor. The autoantibody prevented neointimal regrowth by 65% compared to sham control. The results indicate that when the AT_1 receptor is chronically inhibited by an autoantibody the

regrowth response to arterial injury is significantly reduced. Thus suggests that Ang II and AT_1 receptors are necessary for the growth mechanism in this model of arterial restenosis.

In the antisense inhibition study, we designed AS-ODN targeting to the AT_1 receptor mRNA. We tested it in a well-established model, the central Ang II induced drinking and AVP release. AS-ODN treatment significantly reduced drinking and AVP response to central Ang II injection. The results demonstrate that the antisense inhibition of brain AT_1 receptor gene expression decreases the Ang II induced drinking and AVP response. Thus indicates that antisense inhibition is capable to block AT_1 gene expression.

In order to facilitate uptake of AS-ODN into vessel walls, we tested the dendrimers based gene delivery system in treating restenosis. We investigated the pharmacokinetics and tissue distribution of generation 4,6 and 10 polyamidoamine Starburst® dendrimers. Our results showed dendrimer significantly increased the half life of oligonucleotide in the plasma. We complexed AS-ODN for AT_1 with generation six DEN and tested on a rat model of restenosis. The results showed the AT_1 AS-ODN delivered by DEN significantly reduced neointimal formation. We conclude that vascular RAS and AT_1 play an obligatory role in development of restenosis. The potential of using AS-ODN as a therapeutic method still needs further investigation.

CHAPTER I
INTRODUCTION

Restenosis

Coronary artery disease is the leading cause of death in the United States and in many other countries. The plaques deposited inside the coronary artery, narrow the blood vessel and decrease or completely block the blood supply to the heart. One of the most successful treatments of this disease is arterial angioplasty. The procedure was introduced into the clinic in 1967 by Fogarty. Since then it has become a well established and frequently performed procedure around the world. It is estimated that 350,000 procedures are carried out annually (Hillegass et al. 1994). The procedure has normally an initial success rate of opening obstructed coronary arteries of 95%. However, in spite of the fact that good symptomatic improvement occur in the majority of cases, the procedure is complicated by restenosis in 30-50% of patients, regardless of the type of angioplasty procedure used (Epstein et al 1994). This means more than 100,000 cases of failure and hundreds of million dollars of loss each year.

It is basically accepted by researchers in the cardiovascular field that abnormal growth of the VSMC lining artery walls plays a key role in the blockage of arteries during coronary artery diseases. This abnormal growth also contributes to reblockage (restenosis) of the arteries that have been opened by balloon angioplasty or replaced in

bypass operation The major cause of restenosis is exaggerated healing response of medical VSMC to vascular injury Angioplasty is carried out to restore blood flows to ischemic coronary arteries However, there has always been arterial wall injury inevitably associated with this procedure The injury damages endothelium which normally secretes nitric oxide to prevent VSMC from growth. The injury also stimulates a variety of growth promoters for a repairing procedure (Epstein et al 1994) Growth factors stimulate VSMC to migrate and to proliferate into lumen to form neointima, where they continue to proliferate and secrete extracellular matrix This neointimal mass continues to expand and eventually reblocks the blood vessels (Wilcox1993) Neointimal formation is the result of cell migration, followed by cell proliferation and matrix secretion. At late stage, lumenal narrowing is due to both intimal smooth muscle proliferation and collagen and elastic deposition (Clowes et al 1983) The problem of controlling restenosis becomes largely the problem of controlling the VSMC proliferation

Many factors are involved in regulating of VSMC proliferation Some researchers suggested that most endogenous vasoconstrictive substances are also growth promoters and most endogenous vasodilator substances are growth inhibitors (Dzau 1992) RAS is one of the most important systems involved in restenosis. Traditionally, the renin-angiotensin system is an endocrine system which is involved in regulation of fluid homeostasis and blood pressure (Guyton 1986) The discovery of tissue RAS has led angiotensin research to a new era. Using modern technologies, researchers have found the components of RAS in various tissues, including the blood vessels (Dzau et al 1987) Molecular cloning of the three types of angiotensin receptor subtypes (AT_{1A}, AT_{1B} and

AT_2) allowed us to carry out more intensive studies on their characteristics and their physiological functions (Griendling et al 1993) Understanding of their genetic structure also opened an avenue for gene therapy on angiotensin related diseases For instance, antisense inhibition of RAS has drawn a lot of attention among the researchers.

Pathophysiology of Restenosis

Under normal conditions, VSMC are quiescent During angioplasty, inflation of the balloon causes an increase in lumen size. This gain in lumen size has been shown due to both loss of mass in plaque and overstretch of vessel walls (Clowes et al 1988) Unfortunately, the loss of mass damages endothelial cells, and the overstretch of vessel walls traumatizes VSMC in the media. The direct consequence is to break the balance between the growth promoters and inhibitors. For the side of growth inhibitors, removal of endothelial cells directly causes a reduction in nitric oxide production Nitric oxide is released by endothelial cells in response to the increase of blood flow The immediate effect is to produce vasodilatation and VSMC relaxation (Palmer et al 1987) This will cause an increase in lumen size Apparently, NO is not only a vasodilator but also a growth inhibitor which can prevent VSMC from growing. The intact endothelial cell's layer serves as a screen to stop migration of VSMC into lumen. For the side of growth promoters, a number of growth promoters are produced For instance, Ang II released from endothelial cells and VSMC through a paracrine mechanism begins to stimulate

produced For instance, Ang II released from endothalial cells and VSMC through a paracrine mechanism begins to stimulate VSMC to migrate into lumen leading to cell proliferation there (Dzau 1992) This proliferation is highly exaggerated, then the consequence is reblockage of lumen.

VSMC are highly proliferable cells Cultured rat aortic smooth muscle cells have been used as an experimental model system for the studies of different growth modulators These cells show a high tendency of proliferation even without growth factors' stimulation The cells also have a high density of AT_1 receptor on their membrane surface They are characterized by a high responsive to Ang II stimulation (Rosendorff 1996) The detailed mechanism of how Ang II is involved in restenosis will be discussed in following paragraphs

The Renin-Angiotensin System (RAS)

The classic RAS is an endocrine system which is very important in humoral regulation of the circulation This type of RAS exists in body fluids and has traditional characteristics of hormones Its major component, Ang II, is one of the most powerful vasoconstrictive substances known It is estimated that one millionth of a gram of Ang II can increase the arterial pressure of a human 50 mmHg or more (Guyton 1986) The basic function of this hormone is to cause vasoconstriction, thereby to increase total peripheral resistance and to elevate blood pressure

The Components of the RAS

Figure 1-1 illustrates the components and functional steps by which the classic RAS helps in the regulation of blood pressure. Renin is an enzyme which is synthesized and secreted by juxtaglomerular cells of the kidneys. The function of renin is to cleave angiotensinogen to release a 10 amino acid peptide, angiotensin I. Angiotensin I has no vasoconstrictor. The active vasoconstrictor in the RAS is Ang II which is an 8 amino acid peptide converted from Ang I by ACE mostly in the endothelium of the lungs. Ang II can be inactivated and degraded by angiotensinase. The principal effects of Ang II include vasoconstriction and salt and water retention.

Angiotensin Receptors

There are two major types of angiotensin receptors, AT_1 and AT_2. In rats the AT_1 receptor is further classified into AT_{1A} and AT_{1B} subtypes according to their structure differences. The different Ang II binding sites were first described by their pharmacological characteristics. For example, Ang II type-1 receptor specifically binds Losartan (DuP753) and Ang II type-2 receptor specifically recognizes PD_{123319}. Recent advances in molecular cloning of the cDNAs of these receptor subtypes revealed the true structure different in their genetic level. The cDNAs of the AT_1 receptor were first cloned from rat aortic VSMC and bovine adrenal zona glomerulosa cells (Murphy et al 1991 and Sasaki et al 1991). This type of AT_1 was also recognized as AT_{1A}. Later the other AT_1 subtype, AT_{1B} was cloned from rat adrenal (Murphy et al 1992) gland and pituitary. Two AT_1 subtypes can be also found in mouse genomic DNA, however there is no evidence that the divergence to AT_{1A} and AT_{1B} exists in humans (Smith and

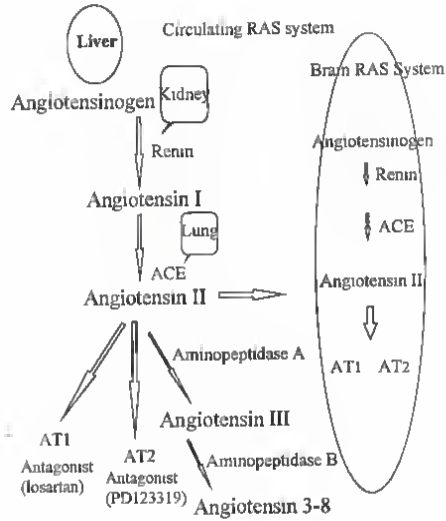

Figure 1-1 Schematic elucidation of the components and functional steps of renin angiotensin system

Timmermans 1994) Rat AT_{1A} is localized on chromosome 17 and AT_{1B} on chromosome 2. In humans, chromosome 3 bears the single AT_1 gene

Rat AT_{1A} and AT_{1B} share about 96% identity in their amino acid sequences However, they have remarkable differences in the noncoding regions which may reflect potential variety in the regulation of gene expression. They are both 395 amino acid proteins with molecular weight of 41 KDa. Hydropathy analysis of amino acid sequences suggests that AT_1 receptor is seven transmembrane domains, G-protein coupled receptor Previous studies have shown that AT_1 receptor is responsible for most traditional Ang II functions

When Ang II binds to its AT_1 receptor, the binding activates a specific protein signaling system These signal transduction pathways include activation of phospholipase C, phospholipase D, calcium channels and other ion channels The signal transduction pathways can be different in different tissues In VSMC, after Ang II binds to AT_1 phosphatidylinositol bisphosphate is hydrolyzed and diacylglycerol and inositol triphosphate are increased During the same time frame, there is a transient increase in intracellular calcium level The immediate consequence of these intracellular signals is the activation of protein kinases, including PKC tyrosine kinases, and a calcium-calmodulin-dependent protein kinase These kinases further phosphorylate a number of other proteins such as MAP kinase, myosin light chain and vimentin, and these proteins mediate cellular functions of smooth muscle, such as contraction

The cDNA of AT_2 receptor subtype was cloned in 1993 from a rat pheochromocytome cell line (PC12w) (Kawamura et al 1993) The AT_2 cDNA

comprises 2,868 nucleotides and encodes a 363 amino acid protein with seven putative transmembrane domains. It shares only 32-35% identical amino acid sequence with the AT_1 subtype, and this identity is mainly concentrated in the putative transmembrane regions. The AT_2 gene has been characterized in humans (Inagami et al. 1995). This gene is located on the X chromosome in both human and rat (Herr et al. 1995). No subtype for AT_2 has been reported.

AT_2 receptor subtype is characterized by its specific binding to PD_{123319} and CGP_{42112A}. This receptor subtype is expressed at very high levels in the developing fetus. By contrast, in the adult its expression is restricted to the adrenal glands, uterus, ovary, heart and specialized nuclei in the brain. AT_2 has also been shown to be a G protein-coupled receptor. Kang et al. (1994) demonstrated that AT_2 modulated K^+ current through Gi. Buisson et al. (1995) showed that AT_2 mediated inhibition of T-type Ca^{2+} current in the NG108-15 cell line through a pertussis-toxin insensitive, G protein. The clear physiological function of AT_2 receptor has not been identified. However, the ping evidence suggested that AT_2 may play a role in some processes such as cellular growth, differentiation or adhesion. Interestingly, although AT_2 disappears quickly after birth in most parts of the body, it can be re-expressed in certain pathological situations involving tissue repair, such as vascular neointima formation and wound healing (Nakajima et al. 1995). Gyurko et al first noted that the AT_1 receptors increase IP_3 hydrolysis and the AT_2 receptors decreased IP_3 hydrolysis in rat skin slices (Gyurko et al. 1992). A report from Dzau's group suggested that AT_2 plays an opposite role against AT_1 in neointima formation after angioplasty (Nakajima et al. 1995). They observed that overexpression of

the AT_2 receptor attenuated neointimal formation in rats. Also in cultured smooth muscle cells, AT_2 receptor transfection reduced proliferation and inhibited MAP kinase activity Tanaka et al (1995) and Yamada et al (1996) suggested that AT_2 could trigger apoptosis in rat ovary granulosa cells and PC12w cells. They further suggested that the mechanism of AT_2 induced apoptosis was mediated by the dephosphorylation of MAP-kinase AS-ODN to MAP kinase phosphatase 1 inhibited the AT_2 receptor-mediated MAP kinase dephosphorylation and blocked the AT_2 receptor mediated apoptosis. Taken together, all these data indicate that AT_2 may play an important role in developmental biology and pathophysiology

There are several selective antagonists for AT_1 and AT_2 receptors Actually the initial classification of AT_1 and AT_2 was based on their different binding characteristics to antagonists Losartan binds to both AT_{1A} and AT_{1B} subtype PD_{123319} is the specific antagonist for the AT_2 receptor These two are also the most frequently used antagonists for the AT_1 and the AT_2 both *in vitro* and *in vivo*.

Tissue RAS

The existence of tissue RAS independent of the circulating RAS, was first described in the early 1970s Tissue RAS occurs in a variety of organs, such as brain, heart, blood vessels and many other organs in the body (Phillips 1987 and Dzau 1987) Modern molecular technology has helped to identify the components of RAS, such as Ao, Renin and Ang II receptors in a large variety of tissues These components were proposed to interact with each other by means of a paracrine and autocrine function. In

the paracrine mode, one cell produces Ang II and delivers it to a neighboring target cell which has receptors to bind and respond to the Ang II stimulation. The autocrine mode describes a cell which produces Ang II, releases it exogenously, and thus feeds back via membrane receptor onto the same cell to regulate the rate of synthesis (Phillips et al 1993).

The brain was among the first tissues that were proposed to have a tissue RAS independent of the circulating RAS. Every key component of RAS has been identified in the brain. Since the brain is protected by the blood brain barrier from circulating Ang II, an independent brain tissue RAS was suggested and thoroughly investigated by many groups (Phillips 1987). Both AT_1 and AT_2 receptors are found in the brain. The AT_1 receptors are distributed in areas associated with cardiovascular effects of central Ang II, such as organum vasculosum lamina terminalis, supraoptic and paraventricular nucleus. The AT_2 subtype are located at locus coeruleus, inferior olive and mediodorsal thalamic nucleus. Its function in brain has not be clearly elucidated. Recently two groups used gene disruption technique to study the possible function of the AT_2 receptor on knockout mice. Hein et al show that AT_2 knockout mice develop normally, but have an impaired drinking response to water deprivation as well as a reduction in spontaneous movements. They also found that baseline blood pressure of the mutants is normal, but they show an increased vasopressor response to injection of angiotensin II (Hein et al 1995). On the other hand, Ichiki et al (1995) reported disruption of the mouse AT_2 gene resulted in a significant increase in blood pressure and increased sensitivity to the pressor action of

angiotensin II. The controversies between the results of two research groups on the AT_2 knockout mice suggest that further investigation will be necessary

There are three distinctive physiological effects of brain angiotensin receptors when Ang II is given centrally The effects are an increase in blood pressure, AVP release and motivation to drink The effects have been shown to be mediated exclusively by the AT_1 receptor, since losartan and the AT_1 AS-ODN blocked these responses (Hogarty et al 1992, Meng et al 1994)

In the vascular system, Re et al (1982) first showed that there was renin in the dog aorta. Since then every major component in RAS has been discovered, including Ao, ACE, AT_1 and AT_2 receptors The AT_1 receptors are located on the membrane surface of medial VSMC and also in the nucleus (Tang et al. 1992) Renin and Ao were found to be at endothelium and media and adventitia. ACE was found in endothelium and some parts of media. AT_1 and AT_2 were both located at medial VSMC The effects of Ang II in the vascular system are twofold In response to Ang II, there are both short and long term effects; vasoconstriction and VSMC growth Stimulation of the AT_2 receptor may cause dephosphorylation of MAP kinase in VSMC (Nakajima et al 1995)

The Renin-Angiotensin System and Restenosis

RAS is an endocrine system which controls body fluid and electrolyte homeostasis Classical RAS is a blood borne circulating hormonal system The target organs for Ang II are the blood vessels, kidney and the adrenal cortex, in which Ang II through its type I

receptor mediates vasoconstriction, decreased glomerular filtration and aldosteron secretion The over all effects will be conservation of water, increased Na^+ reabsorption and increased blood pressure

In addition to circulating RAS, every component of RAS has been found in the vasculature and a paracrine mechanism has been proposed The endothelial cells secrete both NO and Ang II Through a paracrine effect, Ang II and NO are released to act on neighboring VSMC The VSMCs secrete Ang II only Through a paracrine effect Ang II is released on other VSMC Circulating Ang II may also reach the VSMC When the layer of endothelial cells is intact, there is a balance between the growth promoting effect of Ang II and antigrowth effect of nitric oxide, and circulating Ang II probably does not reach the media directly Damage to the endothelial cells, as for example after balloon injury, changes the balance. The growth promoting effects of Ang II become a dominating force. Interestingly, Schwartz suggested that the proliferating VSMC were actually descendants from single colonies of cells that migrated into lumen and these cells in neointima have a stronger response to Ang II than the cells in media (deBlois et al, 1996) They are regulated differently in response to systemic infusion of Ang II Since the VSMC in neointima have also been showed to have a higher density of AT_1 receptor on their membrane, it is very likely that Ang II is one of the early factors involved in VSMC migration (Viswanathan 1993 1994) The mechanism can be proposed as follows After removal of endothelial cells by balloon injury, the damage and distention to VSMC cause an increase in Ang II production and also a upregulation of other components of RAS in the vasculature. The Ang II stimulates the cells that have a higher density of AT_1

receptors to migrate into lumen and the cells begin to proliferate and secrete collagen and elastin. This is a wound-healing process leading to repair of the damage caused by angioplasty. Unfortunately, VSMC can not fully replace the functions of endothelial cells. Instead of NO, VSMCs secrete Ang II. So the repairing process becomes largely exaggerated.

Binding of Ang II to its AT_1 receptor activates a cascade of acute and delayed cellular events. Direct effects include the activation of phospholipase C and generation of metabolites that modulate calcium-sensitive protein kinase C (PKC) activity and cytoplasmic calcium concentration. Ang II binding also activates calcium channels, causing rise to calcium influx to increase VSMC contraction. The PKC mediated protein phosphorylation activates nuclear elements, with long term consequences with regard to gene expression, protein synthesis, mitogenesis and vascular hypertrophy. Thus long term effect of Ang II is very important to restenosis and arteriosclerosis. The mechanism which is proposed by Takeuchi et al. (1990) is as follows. Ang II binding to its AT_1 receptor leads to a rapid increase in *c-fos* and *c-jun* mRNAs levels. The *c-fos* and *c-jun* have been shown to form a heterodimeric transcriptional complex called AP-1 which is able to manipulate target gene expression. This type of Ang II stimulation can be blocked by staurosporine, a PKC inhibitor, or by saralasin, an Ang II receptor blocker. These results indicate that Ang II induced gene expression and cell growth is partially mediated by PKC. On the other hand, calcium has been shown to be important in MAP kinase activation. MAP kinase is also one of the most important growth modulator (Fig 1-2).

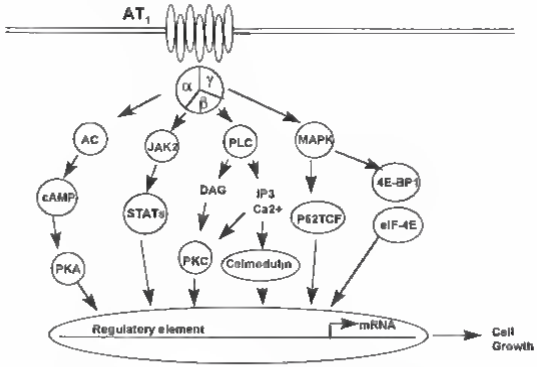

Figure 1-2 Schematic elucidation of the signal transduction of the AT_1 receptor

Experimental Models of Restenosis

To model human responses to angioplasty restenosis and balloon catheter injury, three animal modes are frequently used The most well developed and extensively investigated animal mode of restenosis is the rat common carotid artery The model was introduced by Clowes et al in 1983 In their study, they proposed the VSMC migration and proliferation are the key factors for restenosis The knowledge gained from the rat model has contributed to the understanding and interpretation of the restenosis response in humans However, there are some disadvantages to the current rat model There is no thrombotic component to the response to angioplasty and the rat is resistant to the dietary induction of hypercholesterolemia

The rabbit reinjury system is another widely used animal model Rabbits are fed a very high-cholesterol diet, and primary injury is reduced with a balloon catheter in the iliofemoral artery Six weeks later, the same site is reinjured with a balloon catheter This model allows some thrombosis and rabbits are easy to produce hypercholesterolemia.

Porcine models of restenosis have gained a lot of popularity since their use started in the 1970s. This model is perhaps the best model of restenosis resembling human restenosis The porcine vascular system is very much similar to that of humans The major disadvantage of the pig is its size The pig is more expensive to keep and requires a larger scale in administration of drugs.

Methods to Inhibit Restenosis

Drugs

A large number of different drugs have been tried both on human and laboratory animal models. These drugs include thrombosis inhibitors (aspirin, dipyridamole, enoxaparin, heparin, vapiprost, and warfarin), and VMSC migration inhibitors (cilazapril and heparin). However, so far none of these drugs have producted any significant results in clinical trials.

In 1989 Powell et al first reported that cilazapril, an ACE inhibitor, significantly reduced restenosis in rats when it was delivered via the drinking water for two weeks before angioplasty. After this success, several other research groups demonstrated beneficial effects of ACE inhibitors on different animal models. The AT_1 antagonists, losartan and TCV_{116}, were also reported to be effective (Kauffman et al 1991 and Kawamura et al 1993). However, problems associated with these inhibitors and antagonists (such as side effects, need for repeated administration and the problems associated with dose and time of dosing) have prevented these drugs from effectively treating human restenosis. For instance, although ACE inhibitors have been shown to be effective in preventing animal restenosis in the lab, the human trial by MECATOR failed to confirm any beneficial effects of ACE inhibitors. Later, Rakugi et al (1994) concluded that patients would have to be put on a much larger dose of ACE inhibitors for a longer time in order to inhibit tissue RAS. Traditionally, drugs work on the protein level. Although they can inhibit protein functions, they usually need repeat administration. Non-specific effects and protein upregulation associated with drug inhibition

are frequently observed. Although drug therapies have not solved the restenosis problem in the clinic, they provide us with important insights about mechanisms of this disease. For instance, ACE inhibitors work on both Ang II and bradykinin pathways. They block Ang II synthesis and also enhance NO synthesis. Studies with AT_1 antagonist, losartan, prove that the blockage of Ang II synthesis is the most important part of ACE inhibitor function. Since losartan and TCV_{116}, the AT_1 specific antagonists inhibit restenosis just as well as ACE inhibitors. This result also confirms that the growth promoting effects of Ang II is mediated through AT_1 receptor. Recently Iwai et al. (1997) showed that renin levels in injured blood vessels were increased during the first 3 days after angioplasty and that administration of quinapril significantly reduced neointimal formation. In another experiment they indicated that rat peritoneal macrophage/monocyte cells expressed renin mRNA. Macrophage/monocyte cells may be a source of tissue renin in some pathological conditions (Iwai et al. 1996). They suggested that the upregulation of renin might be the earliest event in vascular RAS activation. These data together show that RAS in vasculature plays an important role in restenosis. Blockage of RAS could be the potential treatment for this disease.

Gene Therapy

Viral vector mediated gene transfer holds great potential in treating restenosis (Table 1-1). There are several vectors available today. They are retroviral vectors, herpes virus vectors adenovirus vectors, and adeno-associate virus vectors. Each vector has its own characteristics. For instance, the retroviral vector will only infect dividing cells. It

works perfectly on cell cultures of dividing cells. However, the efficiency is fairly low when it was used on animals. The herpes virus vector is good for transfecting neuronal cells only. The adeno virus vector has been the most frequently used vector for gene delivery in the vascular system. Its advantages include high efficiency and infectivity for both replication and nonreplication cells. Studies show that the adeno virus vector is able to transfer genes into endothelial and smooth muscle cells. However, the major disadvantage is the immune reaction which it generates. Adeno-associated virus vector is a fairly new vector. It shows great potential for gene therapy. It is non-pathogenic and able to infect most cell types with high efficacy. It incorporates into a genome, so it allows stable and long lasting expression.

The two most significant experiments in gene therapy on restenosis were done by two research groups led by Nabel at the University of Michigan and Leiden at the University of Chicago. Nabel's group transfected balloon injured porcine arteries with an adeno virus vector encoding the herpes virus thymidine kinase (tk). The tk phosphorylates the nucleoside analog ganciclovir and causes DNA chain termination in the transfected cells. The intimal hyperplasia was significantly decreased after a course of ganciclovir treatment (Ohno et al. 1994). Leiden's group used a replication-defective adenovirus encoding a nonphosphorylatable Rb gene. Rb (retinoblastoma) gene is a cell cycle control gene whose nonphosphorylated form is antiproliferative. The nonphosphorylatabe Rb gene product transferred by adeno viral vector significantly blocked restenosis in a porcine femoral artery (Chang et al. 1995). The tk gene transfer method was also repeated in a rat carotid artery model of restenosis and showed results similar to Nabel's report (Guzman et

al 1994) Although gene therapies have many potential advantages over drug therapies in treating restenosis, the safety has always been the biggest problem for this approach The concern has led many researchers to look for alternatives to non-viral gene therapy methods, such as antisense-ODN inhibition.

TABLE 1-1. GENE THERAPY FOR RESTENOSIS

Vector Type	Gene	Animal Model	References
Adenoviral vector	Herpesvirus thymidine kinase (tk)	Pig	Ohno et al *Science* 1994
Adenoviral vector	Retinoblastoma (Rb)	Pig	Chang et al *Science* 1995
Adenoviral vector	Herpesvirus thymidine kinase (tk)	Rat	Guzman et al *PNAS-USA* 1994
Sendai Viral Liposome	Atrial Natriuretic Peptide (ANP)	Rat	Morishita et al *J Clin Invest* 1994

Antisense Inhibition

Since restenosis is largely due to the migration and proliferation of VSMC, the antisense technology makes perfect sense for treatment of restenosis Started in early 1980s, researchers have designed and tested a large number of AS-ODNs targeting numerous growth factors and growth regulatory elements to look for the treatment for restenosis Simons et al (1992) reported that AS-ODN against proto-oncogene *c-myb* gene han antigrowth effects in rat arteries. They used F_{127} pluronic gel to facilitate delivery of AS-ODN and achieved ~80% reduction in neointimal formation Shi (1994) et al showed that proto-oncogene *c-myc* AS-ODN inhibited neointimal hyperplasia using a porous catheter daltvery system in pigs This catheter can infuse AS-ODN solution with

certain pressures into vascular wall to facilitate uptake. Bennett et al (1994) demonstrated that the *c-myc* antisense was also effective in reducing neointimal formation in a rat carotid artery model using F_{127} pluonic gel applied on adventitia. Morishita et al. (1994a) showed that AS-ODN against a cell cycle regulatory enzyme, cyclin-dependent kinase 2 kinase (cdk 2 kinase) gene was able to decrease neointimal formation. Their delivery system was sendai viral liposomes. In their pharmacokinetics study, they showed that the sendai viral liposome could help to retain AS-ODN in blood vessels for 1 week (Morishita et al. 1994a).

TABLE 1-2 ANTISENSE THERAPY FOR RESTENOSIS

Targeting Gene	Delivery System	Animal Model	References
c-myb	F_{127} Pluronic Gel	Rat	Simons et al. *Nature* 1992
c-myc	Transcatheter	Pig	Shi et al. *Circulation* 1994
cdk 2 kinase	Sendai Viral Liposome	Rat	Morishita et al *PNAS-USA* 1993
bFGF	Adenoviral vector	Rat	Hanna et al *J Vasc Surg* 1997

Another antisense approach is to package antisense sequences into expression vectors. Expression vectors will produce antisense mRNA to downregulate gene expression of sense mRNA. For instance, the antisense sequences against (basic fibroblast growth factor) bFGF packaged in an adenovirus vector was shown to be effective in reducing rat restenosis (Hanna et al. 1997). This approach allows the antisense sequence to produced continuously. Antisense inhibition has offered tremendous potential in fighting restenosis. However, the questions, such as which growth factor is the key and which type of AS-ODN is best for patients, still needs to be answered. The delivery

system has always become one of the most difficult hurdles in adopting antisense application.

The Antisense Technology

General

For centuries, researchers have been looking for the "magic bullet"--a drug able to reverse the illness without side effects. Since lots of diseases are caused by overproduction of certain "bad" proteins, most of the work has been focused on protein inhibitors. Recently, however a number of researchers have turned their attention to the genetic level, the machinery which is responsible for producing the proteins. Gene therapy has evolved into a fascinating field which holds great potential to cure diseases, such as AIDS, cancers and cardiovascular diseases. The aims of gene therapy generally fall into two categories, replacement of abnormal genes with normal genes or inhibition of disease causing gene products. Antisense oligonucleotide inhibition belongs to the second category. AS-ODN are specially designed DNA or RNA fragments which are able to interfere with gene expression by binding to DNA or mRNA inside the cells. This new approach for gene manipulation was first proposed by Zamecnik and Stephenson in 1978. In their pioneer experiment for the antisense inhibition, they inhibited Rous sarcoma virus replication with a 13 mer antisense oligonucleotide (Zamecnik and Stephenson 1978). Many researchers have found success using that technique during the past 19 years. Recent discovery of naturally occurring antisense RNA suggests that prokaryotes are actually using antisense RNA in regulating their gene

expression (Wagner et al 1994). It was also proposed by some researchers that, besides microbes, plant and animal cells might also use antisense strategy to control gene expression (Knee et al 1991) In most cases, researchers use short strings of synthetic antisense nucleotides instead of a large antisense genes--although some groups are still working on that. Clinical trials are now in progress for the AS-ODNs in treating several human diseases including acute myelogenous leukemia, HIV infection and CMV (cytomegalovirus) infection (Anderson et al 1996) Isis company has completed its third phase clinical trial for the AS-ODN treatment of CMV infection.

Design of AS-ODN

It is proposed that AS-ODN can work on any of the following processes to block the gene expression. These processes are uncoiling of DNA, transcription of DNA, export of RNA, RNA splicing, and RNA translation. The sequences of AS-ODN are short (usually 15-30 mers) Since phosphodiester ODN has a fast degradation, most AS-ODN in use now is backbone modified. The two most popular modifications are methylphosphonation and phosphorothioation. The methylphosphonation was designed by Ts'o and Miller (1979) They replaced an oxygen atom in each phosphate group with a methyl group (CH_3) This step helped to increase the cellular uptake and provided resistance to break down by enzymes. Phosphorothioates were introduced by Chang et al (1989) They exchanged an oxygen atom with a negatively charged sulfur atom. The phosphorothioates are water soluble and resistant to enzymes. Even now there is no standard rule in selecting target sequences. In general, researchers have found that most regions of the RNA including 5'- and 3'-untranslated, AUG

initiation, coding, slicing junctions and introns can be targeted. The only way to determine which sequence is most effective is through experiments. Wagner (1994) suggests that for any 20 mer phosphorothioate ODN up to 50 sequences should be screened to find an effective AS-ODN. For 15 mer, screening six sequences is efficient to find at lease two sequences to be effective. In our laboratory, we found this is an exaggeration and we have successfully designed useable antisense ODNs by initially designing as few as three sequences.

Mechanism of Action

AS-ODN is theorized to work with at least three different mechanisms. First, AS-ODN can bind to DNA and form a triple helix to block DNA uncoiling and transcription to mRNA. Secondly, AS-ODN can bind to mRNA to interfere with splicing, transporting and translation into protein. Thirdly, AS-ODN can stimulate ribonuclease H (RNAse H) and destroy the DNA-mRNA hybrids. No matter which mechanism is involved, the final result should be a reduction in the protein level, and inhibitory effects on the targeting protein related physiological effects.

Use of Controls

It is always important to use proper controls in experiments to make sure that the effects are real antisense effects. Most frequently used control sequences in antisense research, including sense (S) scrambled (SC), mismatch and inverted. It was also suggested to measure changes of other proteins with similar life cycles along with target proteins. This will show us if the AS-ODN is specifically inhibiting the target protein.

Non-Specific Effects of AS-ODN

Although antisense technology was introduced as a "magic bullet"--a new drug without side effects--the reality is most currently used AS-ODN still can not avoid producing side effects and non-antisense effects, especially when they were used in high concentration. For example, in a cell culture study, we found non-specific cell growth inhibition when the AS-ODN concentration exceeded 25 μM. In order to achieve specific antisense effects, the concentration used must be relative low (<10 μM)

Antisense Inhibition in RAS

Before the introduction of antisense inhibition in RAS, there were already several approaches to inhibit RAS, including ACE inhibitors, renin inhibitors and angiotensin receptor antagonists. However, these drugs are all short acting. In our lab we began to explore the possibility of using antisense strategy for RAS inhibition in 1992. We designed a 15 mer AS-ODN to AT_1 receptor mRNA and a 18 mer AS-ODN to Ao mRNA. Our experiments clearly showed that antisense technology is effective in manipulating gene production in RAS. We found that AS-ODNs against AT_1 receptor significantly reduced the central Ang II induced drinking response when given intracerebraventricularly (Meng et al 1994). The receptor binding study showed the AT_1 AS-ODN decreased AT_1 receptor protein in the hypothalamic region of rat brains. The AS-ODN targeting angiotensinogen (Ao) mRNA significantly reduced high blood pressure in SHR when it was administered centrally and peripherally (Wielbo et al 1995, 1996). Recently we used antisense sequence packaged in an AAV vector and achieved

long term reduction of blood pressure in SHR (Phillips 1997). In summary, all these data suggest that AS-ODN inhibition is effective in RAS gene inhibition.

Summary

Despite the intensive investigation of the role of tissue RAS in the development of restenosis, the controversies remain. It is necessary to use new technologies and novel approaches to further address this problem. Experimental evidence indicates strong connections of the tissue RAS and vascular diseases. In the present study, we exploited autoimmunization and antisense inhibition to investigate the role of RAS in the development of vascular response to injury restenosis. Our results confirmed the important role of Ang II and its AT_1 receptor. Further, we suggest that vascular Ang II is involved in the initiation of the growth response to injury. Antisense inhibition provides a useful tool to study the mechanism involved in vascular injury, and is also a potential therapeutic method for treating restenosis.

CHAPTER 2
HYPOTHESIS AND SPECIFIC AIMS

Hypothesis

There is controversy surrounding the role of RAS in restenosis. The current hypothesis is that Ang II is an initiating and critical factor in response to vascular injury. I will test this by making rats that develop their own autoimmunity to the AT_1 receptor to establish the importance of the AT_1 receptor in restenosis. Second, I will test specific antisense-ODN to AT_1 mRNA for inhibition of AT_1 receptor. Third, I will develop a novel means of delivery of AS-ODN with a dendrimer for potential therapy.

Specific Aims

Specific Aim 1

I will autoimmunize animals against their AT_1 receptor to test specifically and chronically whether Ang II stimulation is critical for the vascular response to injury.

Specific Aim 2

I will test the specific inhibitory effect of AS-ODN targeted to AT_1 receptor mRNA. To accomplish this goal I will use the approach of inhibiting central Ang II effects. Centrally Ang II induced drinking and AVP release will be used as indicators.

Specific Aim 3

I will develop and test a dendrimer based delivery system as an alternative of liposome delivery of AS-ODN *in vivo*

Specific Aim 4

I will inhibit arterial angioplasty induced neointimal formation by inhibition of AT_1 receptor using AS-ODNs delivered by dendrimers

CHAPTER 3
MATERIALS AND METHODS

Experiments of Autoimmunization

Peptide Synthesis and Immunization

Adult male Sprague-Dawley rats (200-225 g) were acquired from Harlan (Indianapolis, In, USA). The animals were kept in individual cages in a room with a 12-hr light, 12-hr dark cycle. They were given tap water to drink and standard rat chow *ad libitum*. Peptide synthesis was carried out at the Protein Core, University of Florida. The peptide sequence was designed corresponding to amino acid sequence 14-23 of the first extra-cellular domain of the AT_1 receptor. The peptide was anchored to polylysine cores to form a multiple antigenic peptides (MAP) according to the method of Tam (1988). This design completely eliminates the coovenuonal step of conjugation of peptides to carriers. HPLC and mass-spectrophotometer were used to check the sequence and purity of the products. For each injection, rat was given 400 µg of peptide mixed with 400 µl of Freund's adjuvant. The animals were immunized with multiple dorsal subcutaneous injections on day 1, 20 and 40.

Animal Model

The animals that produced significant titer (higher than 1000) of antibody after the second injection were used in the experimental group Rats given the same protocol of only Freund's adjuvant injections were used in the control group At day 45, both groups of rats were anesthetized with sodium phenobarbital (30-40 mg/kg body weight) (i.p). Under sterile surgery conditions, a 2 French Fogarty catheter (Baxter Healthcare, Irvine, CA) was introduced into the left femoral artery and threaded through to the left common carotid artery The balloon was inflated in the carotid artery with saline and was passed three times up and down the artery to produce deendothelializing effects (Clowes et al 1988) After surgery animals were returned to cages and kept for two weeks All animals were kept according to the AALAC guidelines for animal care and the experiments were approved by the IACUC committee of the University of Florida.

Cell Culture

Male Sprague-Dawley rats (200-250 g) were acquired from Harlan Rats were anesthetized with phentobarbital and aortas were removed Tissue samples was transferred to 5ml dishes and were digested in 0 67 mg/ml of type II collagenase (Sigma, St. Louis, MO) at 37°C for 30 min After the digestion, adventitia was removed and tissues were incubated with Dulbecco's Modified Eagle's Medium (DMEM) containing 10% of fetal bovine serum (FBS) overnight The following morning, the tissues were digested again with 0 67 mg/ml of type II collagenase and 2 5 mg/ml of elastase (Sigma) for 60-90 min The tissue were triturated with pasture pipettes to speed up the process

The digestion was stopped by diluting with DMEM with 10% FBS Cells were spun for 5 min at 1000 rpm and plated onto culture dishes containing DMEM with 10% FBS

Western Blotting and ELISA for the Antibody Production

Blood was taken from the tail at days 25, 45 and 60 Blood samples (500 µl) were collected in 1 5 ml centrifuge tubes and stored at room temperature for 30 min. Plasma was collected after centrifugation and kept at -20°C until the day of measurement. Polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the method of Laemmli (1970) Membrane proteins extracted from rabbit adrenal glands were amended with 2x sample lysis buffer and boiled for 5 min before loading. After loading the samples on the 10% pre-cast gel (BioRad, CA), the gels were electrophoresed at a constant voltage of 200 V for 45 min on a BioRad Gel Electrophoresis System Rainbow molecular weight standard markers were used in all SDS-PAGEs After electrophoresis, the gels were transferred to PVDF membranes (Bio-Rad) using the transfer buffer systems of Towbin et al (1979), or Szewczyk and Kozlotf (1985) Transferred membranes were blocked for 1 hour in the presence of 5% bovine serum albumin (BSA) at room temperature, then the blots were probed by 1st (rat anti-AT_1 autoantibody) and 2nd (Goat anti-rat IgG alkaline phosphatase, Sigma) antibodies. The antigenic bands were visualized by the incubation in substrate solution (NBT-BCIP)

Quantitation of autoantibody production was determined by ELISA Microtiter wells were precoated with 600 ng/well of the synthetic peptide diluted to 50 mM sodium carbonate-bicarbonate buffer (pH=9 6) and incubated at 4°C overnight. Sodium

carbonate-bicarbonate buffer was added to the microtiter well at the same time to serve as a control. The microtiter wells were incubated at 37°C with 5% milk in PBS for 1 hr before washing five times in washing buffer which was composed of 1x PBS + 0.05% Tween-20. The serum samples containing rat autoantibody were diluted to 1:300, 1:900, 1:2700 and 1:8100 then added to the wells. After 1 hr incubation at room temperature, the wells were washed with washing buffer five times. The optical density was read from a Dynatech 600 microplate reader after the incubation with goat anti-rat IgG complexed to alkaline phosphatase (Sigma) and substrate solution.

Protein Kinase C Assay

Protein kinase C (PKC) assay was carried out by using Calbiochem non-radioactive protein kinase assay kit (Calbiochem-Novabiochem Co., San Diego, CA). Briefly, VSMC cultures were pre-treated with 100 µl of immune serum and control serum in DMEM for 60 minutes. PKC activation was achieved by adding Ang II with final concentration of 100 nM in the culture dishes. After 3 minutes incubation with Ang II (Dixon et al. 1994), incubating solution was asprated off and cells were washed with ice cold PBS and scraped in 0.5 ml of homogenization buffer (20 mM Tris-HCl, 5 mM EDTA, 10 mM EGTA, 0.3% β-mercaptoethanol, 1 mM PMSF, 10 mM Benzamidine). Cells were sonicated for 30 seconds on ice and centrifuge at 100,000 x g for 60 minutes at 4°C. The supernatant was used as the cytosolic fraction. The pellet was resuspended in PBS buffer, plus 0.5% Triton-X-100 for an additional 30 min. The suspension was centrifuged for another 30 min at 4°C at 100,000 x g and was used as the particulate

fraction Protein concentration was determine using Lowry method (Lowry et al 1951) and 500 µg of protein was used in each assay The O D of each well in the assay was read on a Dynatech Immunoassay System at 492 nm.

Serum Transfusion

Blood samples from immunized and control groups were collected after rats were sacrificed Sera were collected after spinning the samples at 1000 x g and stored at -20°C for the future transfusion study Sprague-Dawley rats (300-325 g) were given two infusions of 0 5 ml serum one at 24 hours before surgery and one 1 hr after surgery from the femoral vein The rats was divided into two groups (n=3 each) One group received serum samples from immunized rats, and the other group received serum from control rats. Both groups were subjected to the same procedure of angioplasty after serum transfusion. Rats were returned to their cages and kept for two weeks At the end of the second week, rats were sacrificed and left common artenes were dissected out for the morphological examination

Autoradiography

The method of autoradiography used was described previously in detail (Ambuhl 1995) Briefly, rats were deeply anesthetized with sodium pentobarbital and perfused with 0 9% saline solution intracardially Both carotid artenes were removed and frozen at -10°C for sectioning The sections were cut on a cryostat (20 µm) and mounted onto gelatin-coated slides. The radio-ligand which was used in all experiments was ^{125}I-Sar,Ile-

Ang II. Non-specific, AT_1, and AT_2 binding were determined in the presence of 1 μM of Ang II or PD_{123319} or Losartan. Autoradiograms were generated by exposing the slides to X-ray films for 4 weeks. The photos were taken directly from an image analysis system (MCID, Imaging Research, Ontario, Canada)

Morphological Examinations

Two weeks after balloon catheterization, rats were deeply anesthetized with sodium pentobarbital and perfused via the heart with saline solution. The left and right common carotid arteries were dissected and fixed in 4% paraformaldehyde in PBS for 4 hours. The arteries were sliced at 20 μm thickness transversely on a cryostat machine. The tissues were stained with hematoxylin for the morphological examination. The ratios of the areas of neointima/media were measured and the data expressed as a ratio of intimal/media areas.

Immunohistochemistry

The immunohistochemical staining includes two separate experiments. In the first experiment, we applied rat autoantibody to recognize the AT_1 receptor on the rabbit. These aortas were from atherosclerotic rabbits in which the AT_1 receptor is highly upregulated in the aortas (Yang et al 1997). The second experiment involved using a rabbit polyclonal antibody to AT_1 receptor to stain the carotid arteries from both immunized rats and control rats. The immunohistochemistry was carried out by using an ABC peroxidase staining kit (Pierce Chemical Company, USA). Briefly, tissues were

sliced into 30 µm sections on cryostat, after an overnight incubation in 1x PBS at 4°C overnight. After incubation with 1st and 2nd antibody, AT_1 signals were visualized by exposing sections to 3,3-diaminobenzidine

Experiments on Central Ang II Inhibition

Animals

Adult male SHR and Sprague-Dawley (SD) rats (250-300 g) were acquired from Harlan (Indianapolis, IN USA). The animals were kept in individual cages in a room with a 12 h light-12h dark cycle. They were given tap water to drink and standard rat chow to eat *ad libitum*.

Surgery

Rats were anesthetized with sodium pentobarbital (30-40 mg/kg body weight i.p) and a stainless steel, 23-gauge cannula was placed in the right lateral ventricle, using a Kopf stereotaxic instrument (stereotaxic coordinates: 1.0 mm lateral, 1.0 mm caudal to the bregma, 5.0 mm below the skull surface). The cannula was anchored with stainless steel screws in the skull and covered by dental acrylic. A steel wire obturator was placed in the cannula to maintain patency. Animals were returned to home cages to recover from surgery for 5 days. Five days after surgery a catheter filled with heparin (100 U/ml) was placed in the left common carotid artery under sodium pentobarbital (30-40 mg/kg body weight i.p.) anesthesia for blood sampling. The experiments were performed 24 hours after the carotid catheterization. The

obturator was removed and a 30 gauge injector, connected to a Hamilton microliter syringe (no 700) by silastic tubing, was inserted in the guide cannula. (1) Ang II (50 ng) dissolved in 2 µl of artificial cerebrospinal fluid (ACSF) was injected. This injection was used to establish the control responses and to verify to cannula placement (a positive drinking response is a good indication that the cannula is in the ventricle) It has been shown by Hogarty et al (1992) that ACSF has no effect on drinking or AVP release when given centrally The dose of 50 ng/Ang II was established by prior dose-response studies in this laboratory (2) One hour after the Ang II injection, 50 µg antisense ODN targeted to the AT_1 receptor, or scrambled ODN as control dissolved in 4 µl isotonic saline was injected i.c.v into the lateral ventricles. (3) Ang II (50 ng) (i.c.v) was administered 24 hours later to test the degree of inhibition by AS-ODN (4) The experiments were performed in both Sprague-Dawley and SHR groups. In two Sprague-Dawley groups, the rats also received 2 i.c.v injections at 24 h intervals with 50 µg of antisense, or scrambled ODN or 4 µl isotonic saline.

Oligodeoxynucleotides

Antisense (AS) oligodeoxynucleotides was synthesized as 15-mer to bases +63 to +77 of angiotensin II type 1 receptor mRNA. Scrambled control ODN was a 15 mer with a random sequence of the same bases. The ODN was modified by backbone phosphorothioation. ODNs were synthesized in the DNA Synthesis Laboratory, University of Florida, Gainesville, FL. The sequences were as the follows AS 5' -TAACTGTGGCTGCAA-3', Sc 5' -AATTGGTGTGTTTCGTTC-3'

Vasopressin Assay

Vasopressin assay was performed according to the procedure of Hogarty et al (1992) Briefly ODN treated rats were injected i.c.v with Ang II (50 ng), and blood samples for the vasopressin assay in plasma were drawn at 1 min after injections. Blood samples (1-2 ml) were collected in chilled tubes containing 0.3 M ethylenediaminetetraacetic acid (EDTA) (50 µl/ml blood) from the carotid catheter Plasma was collected after centrifugation and stored at -20°C until the day of extraction. The assay used was based on that of Raff et al (1991) Plasma AVP was measured using an antibody, raised in rabbits, against AVP Cross-reactivity with other hormones (e.g. oxytocin, vasotocin, angiotensin I angiotensin II) was <0.001% AVP was extracted by absorption to bentonite with an 80% recovery Plasma (0.5 ml) was extracted and then reconstituted by using assay buffer Radiolabeled iodide [^{125}I]AVP (DuPont) was used as the tracer and AVP (sigma) was used as a standard The detection limit of the assay was 0.078 pg/tube.

Drinking

Drinking was measured for 30 min after Ang II injection. The water intake was read directly from scaled drinking bottles and is expressed as ml/30 min.

Radioligand Binding Assay

Sprague-Dawley rats were given three i.c.v injections of antisense, or scrambled ODN (50 µg each injection) or 4 µl isotonic saline at 24 h intervals. Rats were killed by decapitation 6 h after the third injection and the hypothalamic block including the hypothalamus, thalamus

and septum was dissected Membrane proteins (100 µg) extracted from the hypothalamic block were used in the binding assays [^{125}I]SI-AII (0 2 nM) was used as radioligand in all experiments. The total volume for each tube is 500 µl. The incubation time was 90 min at room temperature Ang II and losartan (1µM each tube) were used to determine specific binding and AT_1 receptor binding respectively

Experiments on Dendrimer Delivery System

Dendrimer Labeling and Purification

Dendrimer generations four (Aldrich Inc.), six (Polysciences Inc.), and ten (Dendritec Inc.) were labeled by using fluroecein isothiocyanate (FITC) (Molecular probe) The conjugation procedure was carried out in a centrifuge tube with continuous stirring for 24 hours at room temperature in a 1 to 1 1 molar excess of the isothiocyanate G-10 Sephadex (Sigma) spin columns were used for the separation of FITC labeled dendrimer from unreacted FITC. Labeling of dendrimers was confirmed by analyzing the Den samples by thin layer chromatography (TLC) on Whatman PE Sil G/UV plates with a mobile phase of chloroform, methanol ammonia (7 2 1)

Dendrimer and Oligonucleotide Conjugation

To determine if the dendrimers are be able to complex with oligonucleotides and form relatively stable compounds, we tested the DEN's ability to cause ODN-gel retardation on a 15% horizontal non-denaturing polyacrylamide gel system in TBE buffer

FITC-labeled 15 mer antisense oligonucleotide targeting rat AT_1 receptor mRNA were used in the conjugation studies with the dendrimers. The 6th generation dendrimers were complexed to the phosphorothioate antisense oligonucleotide by mixing them together in PBS buffer. The molar ratios of oligonucleotide to dendrimers were 1:1, 1:0.5, 1:0.1, 1:0.01 and 1:0 respectively. The samples were loaded into wells after premixed with 50% glycerol. The photos were taken under UV transluminator at 360 nM.

In vivo Metabolism

Male Sprague-Dawley rats weighing 200-250 g were anesthetized with 100 mg/kg of ketamine and 2 mg/kg of xylazine intraperitoneally (i.p.). The left femoral vein was cannulated and injected with 0.5-1.0 ml of sample with contained either FITC labeled dendrimer or DEN complexed with FITC-ODN. The right femoral artery was cannulated with a PE-50 plastic catheter, and blood samples (100 µl) were collected at 1, 3, 5, 10, 15, 20, 30, 60 min after a single intravenous infusion of sample. Blood volume was replaced with same amount of physiological saline solution infused from femoral vein during the entire experiment. The blood samples were kept at room temperature for 1 hour, then centrifuged at 1000 g for 30 min. Serum (50 µl) was diluted with PBS (450 µl). FITC signals in plasma were determined at wavelength 487 nm (ex) and 525 nm (em) by using a Perkin Elmer LS50B Luminescence spectrophotometer.

Tissue Distribution of Dendrimer Based Delivery System for AS-ODN

Twenty-four hours after sample infusion, rats were sacrificed and tissues including brain, kidney, liver, skeletal muscle and blood vessels (aorta and common carotid artery) were removed The tissues were sliced to 20 μM sections and mounted to glass slides. The FITC signals were visualized by using confocal microscopy

Antisense Inhibition of Restenosis

Sprague Dawley rats whose body weight range from 300-375 were acquired from Harlan (Indianapolis, IN) Rats were anesthetized with sodium pentobarbital (30-40 mg/kg body weight) (i p) and a 2 French Fogarty catheter (Baxter Healthcare,) was introduced from left femoral artery to the left common carotid artery The balloon was inflated by saline and was passed three times up and down at left common carotid artery to produce deendothelialzing effects After balloon catheterization, another infusion catheter was introduced from left external carotid artery into left common carotid artery The artery segments were tight up in order to retain the substances released from infusion catheter (Fig 3-1) Rats received 100 μg of AS-DEN (antisense), SC-DEN (scrambled) or DEN (dendrimer only) of infusions at certain pressure to facilitate uptake into vessel walls. After surgery, animals were returned to cages. Animals were scarified at different time points ranged from 7 hours to 7 days Rats are anaesthetized and perfused with 0 9% saline solution intracardiacliy Both carotid arteries were removed and frozen at -10°C for sectioning The sections were cut on a cryostat (20 μm) and mounted onto gelatin-coated slides.

Statistics

All values are expressed as mean ± S E M. Data were analyzed by using ANOVA or student t-test, followed by the Duncan multiple range test. In all analyses, a P value of less than 0 05 was considered significant.

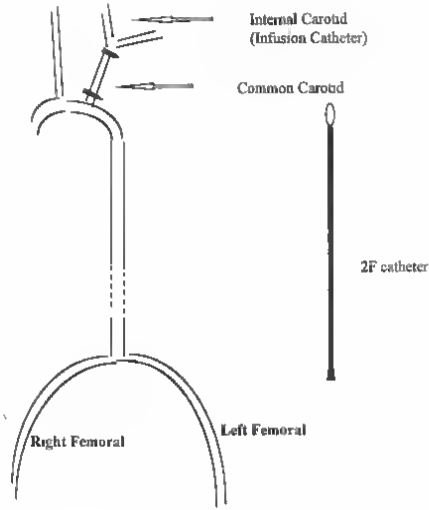

Fig 3-1 Schematic elucidation of the procedure of balloon catheterization on rat carotid artery

CHAPTER 4
AUTOIMMUNIZATION AGAINST ANGIOTENSIN TYPE-1 RECEPTOR PREVENTS THE NEOINTIMAL PROLIFERATION FOLLOWING ANGIOPLASTY

Introduction

Arterial angioplasty is one of the major therapeutic methods to treat the ischemic coronary heart diseases. However, arterial injury associated with this procedure results in neointimal formation, which eventually causes restenosis in 30-50% of patients (Hillegass et al. 1994, Popma et al. 1991). The restenosis is mainly due to overgrowth of vascular smooth muscle cells (VSMC). Under normal physiological conditions these cells are non-proliferative due to suppression by endothelial cells lining the vessel lumen (Palmer et al. 1987, Kinsella et al. 1986). Arterial angioplasty destroys the endothelial cells of arteries and removes the growth inhibitory function of endothelial cells. The procedure also stimulates activation of growth factors on VSMC in the media. This causes VSMC to begin to migrate and proliferate into the lumen resulting in neointimal formation (Wilcox et al. 1993). Eventually overgrowth of neointima reduces lumenal size and blocks blood flow. Many factors have been shown to be involved in this process. Among those factors angiotensin II has been suggested to promote restenosis (Wilcox et al. 1993, Gibbons et al. 1994, 1996, Powell et al. 1989). The vascular wall is one of the many tissues that has been proposed to have a local renin angiotensin system (RAS), independent from plasma

RAS (Dzau et al 1987) Ang II functions through its specific receptors on the cell membrane. There are two main sub-types of Ang II receptors, AT_1 and AT_2. The AT_1 is responsible for vasoconstriction and the growth effects of Ang II on blood vessels (Wilcox et al 1993) The AT_2 subtype has been shown to have anti-growth effects (Nakajima et al 1995) The binding of angiotensin II to AT_1 receptor triggers a cascade of intracellular events leading the activation of phospholipase C and generation of inositol triphosphate (IP_3) and diacylglycerol (DAG) (Griendling et al 1994) IP_3 is responsible for the increase of intracellular calcium level and DAG stimulates protein kinase C (PKC) activation. Both pathways have been shown to be important for VSMC growth (Duff et al 1995)

The attempts of using angiotensin inhibition to treat restenosis began with the study of Powell et al in 1989 In their study, they administered ACE inhibitor, Cilazapril to the balloon injured rats and achieved 80% reduction in neointimal formation (Powell et al 1989) In the following years several studies cases have been reported using variety of ACE inhibitors and AT_1 antagonists to prevent restenosis in animal models (Kauffman et al 1991, Taguchi et al 1993, Kino et al. 1994) However, despite the extensive study of the role of the renin-angiotensin system in development of restenosis, controversies remain In clinical trials, we still do not have a clear picture on the importance of angiotensin in development of restenosis The MERCATOR (Multicenter European Research trial with Cilazapril after Angioplasty to prevent Transluminal Coronary Obstruction and Restenosis) trial failed to confirm a beneficial effect of ACE inhibition in human subjects (Hermans et al. 1993). However, Yamabe et al (1995) showed that the treatments with cilazapril 7 days before angioplasty significantly reduced the rate of

restenosis in human. Rakugi et al (1994) later suggested in their study that higher dose of cilazapril may be need to inhibit tissue RAS. When we review the protocols used, we conclude that most of the difficulties are due to the dose and time of dosing with ACE inhibitors. Apart from the protocol decision of when to and how much to administer ACE inhibitors, there is the possible response of upregulation of receptor or inadequate reduction of Ang II at a critical time. It is beneficial for us to go back and carefully review the results of the animal studies. The condition that appears to have been important to treatment with ACE inhibitors prior to injury (Powell et al. 1989), whereas in the MERCATOR trials cilazapril was given after injury. Utilization of new and novel techniques will also be helpful to explore this problem.

As a useful technique to explore protein functions, autoimmunization overcomes the difficulties of drug therapies, such as problems with dose and time of dosing (Sooa et al. 1995, Fu et al. 1996). Autoimmunization is chronic and complete. In the present study, we designed an experiment to induce rats to produce autoimmunity against the N-terminal of AT_1 receptor. The N-terminal peptide is the first extracellular loop of the 7 transmembrane receptor and was shown to be important for PKC activity induced by Ang II (Vinson et al. 1994). The immunized rats were then subjected to balloon injury of the carotid artery. We hypothesized that if Ang II is critical in the vascular response to balloon injury, blocking Ang II function with a specific autoantibody would prevent VSMCs proliferation and achieve a reduction on neointimal formation. To further test our hypothesis, we also transfused antiserum containing AT_1 autoantibody into normal rats

and performed balloon injury on these recipient rats In both experiments we achieved a significant reduction of neointimal proliferation

Results

Over 50% (11 out of 20 rats) of the immunized rats produced a significant amount of autoantibody in the ELISA screening (with the titers of 1000-2000) after the second injection These animals were used as immunized group for the balloon catheterization experiments

Western blot analysis of the protein extracts from rabbit adrenal glands using the AT_1 antiserum shows a single band with molecular weight of 65 kDa (Fig 4-1) This result corresponds well with the molecular weight of the mature glycosylated AT_1 receptor (Barker et al 1993, Desarnaud et al 1993) Occasionally two other minor bands with molecular weights of 43 and 55 kDa were also observed These two bands may be the result of deglycosylation during extraction procedures The 43 kDa is the predicted molecular weight of unglycosylated AT_1 protein (Murphy et al 1991, Desarnaud et al 1993) This result indicates that the antigen induces a specific autoantibody against the AT_1 receptor protein in immunized rats

PKC assay was carried out on cultured VSMC Ang II (100 nM) stimulated a rapid translocation of PKC from the cytosol to the membrane in 3 minutes (Fig 4-2) After 60 minutes incubation with immune serum, the Ang II induced PKC translocation was significantly reduced, while the incubation with control serum had little impact on PKC translocation

There was staining with the autoantibody of rabbit AT_1 receptor Figure 4-3 shows immunohistochemistry of staining AT_1 protein on rabbit aorta with antiserum from the immunized rats. The autoantibody specifically recognized AT_1 receptor on VSMCs in media. The result of staining for AT_1 receptors in the autoimmunized rats showed the presence of autoantibody blocked exogenously applied antibody from binding. There was no staining on adventitia. Fig 4-4b shows that the carotid arteries from immunized rats could not be stained using a rabbit AT_1 polyclonal antibody The control rats which were injected with only Freund's adjuvant showed obvious staining to the rabbit AT_1 antibody (Fig 4-4a) Autoradiographies of multiple transverse sections of carotid arteries showed that the location of AT_1 receptor is in the VSMCs (Fig. 4-5a) The AT_1 receptor binding was decreased on the sections of immunized rats (Fig. 4-5b)

The effects of AT_1 autoantibody on restenosis are shown in Figure 4-6 Autoantibody produced significant inhibitory effect on neointimal formation Morphological analysis of the cross sections by ratios of neointimal/medial areas shows that the immunized group had significantly lower neointimal growth than the control group ($p<0.01$)(Fig. 4-7).

In order to rule out the possibility that metabolic changes associated with autoimmunization may contribute to the growth inhibition, we also carried out the blood transfusion study In the experiment, we transfused the sera collected from control or immunized rats to two groups of new rats at the onset of blood catheterization The rats that received twice immune serum injection (1 ml each) showed significant lower

neointimal formation when compared to of those received same amount of control serum ($p<0.05$) (Fig 4-8)

Discussion

In the present study, we demonstrated that the neointimal formation induced by balloon angioplasty could be inhibited by actively inducing autoantibody production against the N-terminal of the angiotensin II type-I receptor We also showed that transfusion of antiserum during early stage of balloon injury reduces arterial regrowth Our results support the hypothesis that Ang II is a critical growth promoter in the vascular system in response to injury Our results further suggest that Ang II is one of the early growth factors in the process of restenosis. The present study offers the first report that an antibody against the N-terminal of the AT_1 protein inhibits neointimal formation.

The primary pathophysiologic mechanism in restenosis is the migration and proliferation of VSMCs in the subintimal layer (Clowes et al. 1983), where they form neointima and decrease the lumenal diameter Arterial angioplasty removes the endothelial cells, which secrete nitric oxide and heparan sulfate proteoglycan that apparently maintain VSMCs in a quiescent state (Palmer et al 1987, Kinsella et al 1986) The distention in the procedure of angioplasty traumatizes VSMC and stimulates the production of growth promoting factors (Clowes et al 1989) Blood vessel is one of the tissues that have been shown to have a local renin-angiotensin system (RAS) (Dzau et al. 1993) Gibbons et al (1992) show that Ang II have growth effects on VSMC and the growth actions are

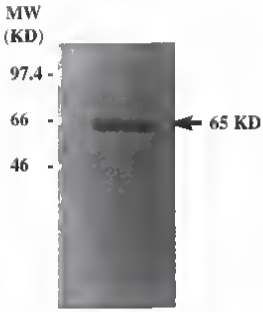

Fig 4-1 Western-blot analysis of membrane proteins from rabbit adrenal glands using antiserum from immunized rats Membrane proteins extracted from rabbit adrenal were loaded on the 10% pre-cast gel and were electrophoresed at a constant voltage of 200 V for 45 min The gels were transferred to PVDF membranes, then the blots were probed by 1st (rat anti-AT_1 antibody) and 2nd (Goat anti-rat IgG alkaline phosphatase) antibodies. The antigenic bands were visualized by incubating in substrate solution (NBT-BCIP) There was a single band with MW of 65 kDa recognized by the rat antibody

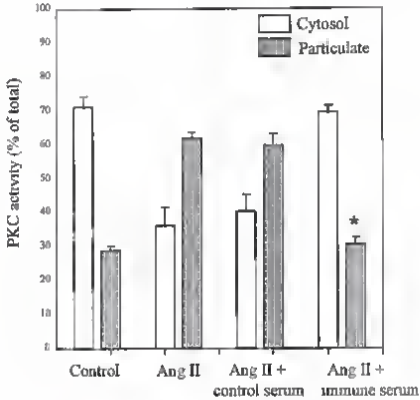

Fig 4-2 The effect of immune serum containing autoantibody to the N-terminal of the AT_1 protein on PKC translocation Cultured VSMCs were pre-incubated with immune and control serum for 60 minutes 100 nM of Ang II was used to induce PKC translocation Ang II induced a rapid increase in membrane-bound (particulate) PKC activity accompanied by a reduction in cytosolic PKC level The pre-incubation of immune serum resulted in a significant blockage of Ang II induced PKC translocation ($P<0.05$ $n=3$), while control serum had no significant impact on PKC

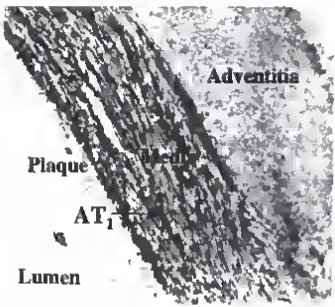

Fig 4-3 Immunohistochemical identification of AT_1 receptor on the sections of rabbit arteries using the rat AT_1 autoantibody The immunohistochemistry was carried out by using an ABC peroxidase staining kit (Pierce Chemical Company, USA) Tissues were sliced into 30 μm sections on cryostat After incubation with 1^{st} (rat AT_1 autoantibody) and 2^{nd} (Goat anti-rat IgG) antibodies, AT_1 signals were visualized by exposing sections to 3,3-diaminobenzidine The rat AT_1 autoantibody located most of the AT_1 receptor on medial smooth muscle cells

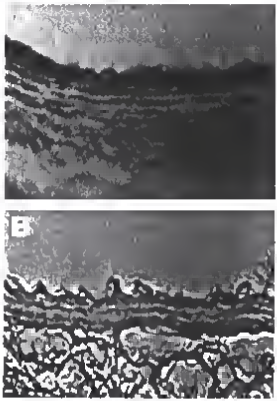

Fig 4-4 Immunohistochemical staining of sections from rat carotid arteries using a rabbit polyclonal antibody against AT_1 receptor The immunohistochemistry was carried out by using an ABC peroxidase staining kit (Pierce Chemical Company, USA) The 1st antibody was the rabbit polyclonal antibody against AT_1 and the 2nd antibody is goat anti-rabbit IgG provided with kit AT_1 signals were visualized by exposing sections to 3,3-diaminobenzidine A Representative section of a carotid artery from a injured normal control rat exhibited intense staining of AT_1 receptor on neointimal and medial VSMCs B The artery of autoantibody producing rat was not be able to be stained using the same rabbit antibody and experimental protocol

Fig 4-5 ^{125}I-Sar,Ile-Ang II autoradiography analysis of multiple transverse sections of carotid arteries The sections were cut on a cryostat (20 μm) and mounted onto gelatin-coated slides The radio-ligand which was used in all experiments was ^{125}I-Sar,Ile-Ang II Non-specific, AT_1 and AT_2 binding were determined in the presence of 1 μM of Ang II or PD_{123319} or Losartan Autoradiograms were generated by exposing the slides to X-ray films for 4 weeks The photos were taken directly from an image analysis system (MCID, Imaging Research, Ontario, Canada) The pictures represent the specific binding of AT_1 receptor A, representative section from control rats B representative section from immunized rats

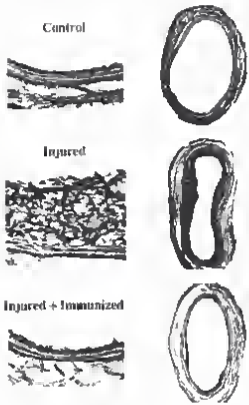

Fig 4-6 Photomicrographs of representative histological sections from sections of rat left common carotid arteries 2 weeks after balloon injury Two weeks after balloon catheterization, rats were deeply anesthetized and perfused via the heart with saline solution The left and right common carotid arteries were dissected and fixed in 4% paraformaldehyde for 4 hrs. The arteries were sliced at 20 μm thickness transversely on a cryostat machine The tissues were stained by hematoxylin for the morphological examination A,B Uninjured C,D Injured E F Injured and immunized

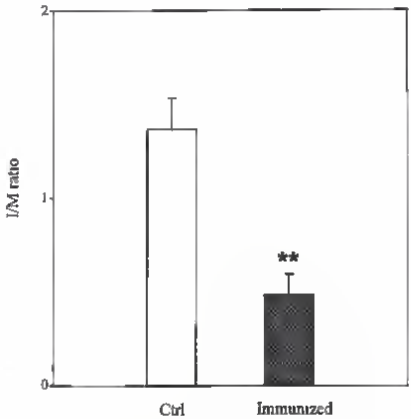

Fig 4-7 Bar graphs represent the ratios of intimal/medial areas in the two groups of rats that underwent balloon catheterization Values are expressed as mean ± SE Data were analyzed by Student t-test A p value of less than 0 05 was considered significant (*) and a p value less that 0 01 was considered highly significant (**) The immunized rats (n=6) showed highly significant ($p<0\ 01$) smaller regrowth ($0\ 49 \pm 0\ 11$) than the control group ($1\ 37 \pm 0\ 17$) (n=6)

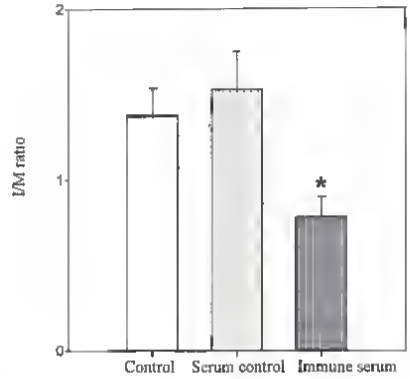

Fig 4-8 Bar graphs represent the ratios of intimal/medial areas in three groups of rats that underwent balloon catheterization Values are expressed as mean ± SE. Data were analyzed by ANOVA followed by the Duncan multiple range test A p value of less than 0 05 was considered significant (*) The rats received immunized serum (n=3) demonstrated significant less neointimal regrowth (0 78 ± 0 12) than those of two control groups [(1 37 ± 0 17) for the control rats (n=6), and (1 53 ± 0 22) for the rats received control serum (n=3)]

mediated by the type I receptor (AT_1). *In vitro* studies on VSMC cultures indicate that Ang II stimulates cellular proto-oncogenes (eg *c-fos* and *c-jun*) that are important in the regulation of cell growth (Naftilan et al 1989, Lyall et al 1992) These effects can be inhibited by losartan, an AT_1 specific antagonist In *in vivo* studies, chronic infusion of Ang II results in vascular hypertrophy (Griffin et al 1991) Transfection of blood vessels with ACE gene causes neointimal formation (Morishita et al 1994) Indirect evidence for the involvement of the RAS first came from the observations that neointimal formation could be prevented by ACE inhibitor or AT_1 antagonist administration (Powell et al 1989, Kauffman et al 1991, Taguchi et al 1993, Kino et al 1994) Interestingly, VSMCs in neointima have been shown to have stronger response to Ang II than those in media (deBlois et al 1995) Since the VSMCs in neointima have also been shown to have higher density of AT_1 receptor on their membrane than those in the media, it is very likely that Ang II is one of the early factors involved in VSMC proliferation and migration (Viswanathan et al 1992)

The method of using the synthetic peptide induced autoantibodies has been well documented in studies of autoimmune diseases (Fu et al. 1996, Soos et al 1995) Synthetic peptides induce animals to produce antibodies that react with their cognate sequences in the native protein We took advantage of multiple antigen peptides (MAP) in our study MAP are highly branched polylysine molecules which have capacity to complex peptides on their surface. They do not induce immune responses themselves (Tam et al 1996) When they complex with antigenic peptides, the conjugates are able to induce specific immune response to the peptides Numerous reports have confirmed that

MAP induce specific immune responses to the antigens coupled to them without any side effects (Tam et al 1996) The immune response alone could not be responsible for the inhibition of neointimal formation since both immunized rats and rats treated with antiserum showed the similar effect of inhibition.

The specificity of the antibody was shown by using Western blotting. A 65KDa protein was specifically selected by the autoantibody This is the correct molecular weight of mature glycosylated AT_1 receptor (Desarnaud et al 1993) The antibody positively stained AT_1 receptor on rabbit aortas This is more evidence that the autoantibody is capable of binding AT_1 receptor in the vascular system Further proof was shown by the carotid arteries from the immunized rats which could not be stained by a polyclonal AT_1 antibody raised in rabbit The lack of staining by the rabbit antibody imply preoccupation of the AT_1 receptor by autoantibody Immunohistochemistry showed dense staining of AT_1 receptor in the neointima. This result is consistent with the report from Viswanathan et al (1992) Their study showed that neointimal VSMCs have higher density of AT_1 receptors using autoradiography It is possible that the VSMCs in the neointima are regulated differently from those in media (deBlois et al 1995) The VSMCs with higher AT_1 receptor number and stronger response to Ang II stimulation may first migrate into the lumen, where they form neointima by replicating themselves. The cells in neointima may be the decedents of this particular type of VSMCs that have more AT_1 receptors In this study, immunized rat vessels showed less receptor binding sites in autoradiography There are three possible explanations First, the autoantibody interfered with Ang II binding. Secondly, the density of AT_1 receptor was decreased due to the growth

inhibitory effect of the autoantibody on VSMC Third, although Vinson et al (1995) suggested that the antibody to the N-terminal part of the AT_1 receptor does not interfere with Ang II binding, the autoantibody may act as a false signal of ligand binding which leads to down regulation of the receptor by negative feedback

Ang II acts on VSMCs through the activation of phospholipase C, which catalyses the breakdown of phosphatidylinositol-4,5-bisphosphate to IP_3 and DAG The main role of DAG is to activate PKC Activation of PKC is one of the most important events in the AT_1 signaling cascade. It is also suggested to be a important pathway mediating Ang II induced growth effects in VSMCs (Takeuchi et al 1990) We chose the PKC activation as the indicator for the inhibitory effect of autoantibody on the AT_1 receptor into presence of Ang II The ability of the autoantibody to interfere with physiological function of the AT_1 receptor was confirmed by PKC assay on cultures of VSMC Activation of PKC in VSMCs features a rapid translocation PKC from the cytosol to the cell membrane This event happens in 1-5 minutes (Haller et al 1992, Dixon et al 1994) Showing that Ang II induced PKC translocation can be blocked by the antibody on VSMC cultures, we further confirmed the capability of the autoantibody to inhibit signal transduction of the AT_1 receptor

The immunized rats had significantly lower neointimal growth after balloon injury This is direct physiological evidence that the AT_1 antibody attenuates cell proliferation. Our result is further strengthened by showing that the serum from immunized rats is able to inhibit neointimal formation when it is transfused into normal rats. The results are critical for the specificity of the antibody effect. It excludes the possibility that a

nonspecific increase in immune response might be attributed to the inhibitory effects on neointimal formation Interestingly, we achieved significantly inhibitory effects by only two infusions of antiserum The first infusion was at 24 hours before the balloon catheterization, and the second one was on the day of surgery This experimental protocol is different from those of Ferns et al (1991) who infused a PDGF antibody and from Lindner et al (1991) who infused an bFGF antibody. They continuously boosted their animals with antibody until the animals were sacrificed Our result may indicate that Ang II is one of the early responding factors to the vascular injury and leads us to hypothesize that down-regulation of Ang II function at the early stage of injury is able to attenuate the initiation of restenosis

There are limited data on time course studies either with ACE inhibitor or with AT_1 antagonists in the animal models of restenosis In most cases of ACE inhibitors, experimental animals were put on drugs several weeks before the angioplasty and drugs were continuously available during entire period of the development of restenosis (Powell et al 1989) However, losartan was tested after angioplasty (Kauffman et al 1991) It was reported by Prescott et al (1991), that ACE inhibitors inhibit the migration of VSMCs only, losartan, however, affect both migration and proliferation. This interesting phenomena may explain why ACE are ineffective when they are administered after angioplasty (MECATOR 1994) Vascular RAS has been proposed to one of the early factors involved in initiation of restenosis (Dzau et al 1993), but since there are many growth factors such as PDGF, bFGF and many cell cycle genes are involved the timing of the role of Ang II is not clear In present study we support the hypothesis by showing that

AT_1 auto antibody significantly blocked restenosis and was effective when transfused at the initiation of the response to injury

Autoantibody inhibition is obviously not meant to be a practical approach to preventing restenosis clinically, however it provides a powerful tool to explore the role of the factors that are involved in this complicated process. The experiments have been carried out in rats There is a debate whether that rat is a useful model for human restenosis The debate was fueled by the failure of angiotensin converting enzyme inhibition to reduce restenosis in humans as they had done in rats However the protocols used in patients were different from the protocol used in rats. The pig has been used as an alternative model, but treatment for restenosis has not been transferred from porcine studies to the clinic. Since the rat is available and the problems of restenosis are complex, the rodent model still offers a fruitful substrate for unraveling some those complexities Based on this study with autoimmunity to AT_1 receptors. We conclude that Ang II and the AT_1 receptors are involved in the initiation of growth mechanism in response to vascular injury

CHAPTER 5
ANTISENSE OLIGONUCLEOTIDE TO AT_1 RECEPTOR mRNA INHIBITS CENTRAL ANGIOTENSIN INDUCED THIRST AND VASOPRESSIN

Introduction

Central injection of angiotensin II (Ang II) elicits several distinct physiological responses including an increase in blood pressure, vasopressin release, natriuresis, salt appetite, and a motivation to drink (Phillips et al 1987). Since the brain is protected from blood-borne Ang II by the blood-brain barrier, the existence of a brain renin-angiotensin system (RAS), independent of peripheral RAS was proposed (Ganten et al. 1983). All components of the RAS have been identified in brain (Deschepper et al 1986, Dzau et al 1986, Lynch et al. 1986, Phillips et al 1985 and Unger et al 1991). Although it is still not clear how the components interact, a paracrine action has been proposed (Phillips et al 1991). There are at least two types of Ang II receptors which have been found in brain. AT_1 receptors are located in the brain regions which are involved in cardiovascular control mechanisms (Aldred et al 1993, Phillips et al 1985). Specifically blood pressure, drinking, and AVP release are mediated by the AT_1 receptor (Hogarty et al 1992, Kirby et al 1992, Timmermans et al 1992). AT_2 receptors are located in specific areas such as the cerebellum, the inferior olivary nucleus, the locus coeruleus and the thalamus (Wright et al 1994). The role of AT_2 receptor still need further investigation. Recently, Huang et al (1996) suggested that in rat brain neuronal cultures, MAP kinases were inhibited by the AT_2 receptor stimulation being stimulated by AT_1 receptor

activation. Spontaneously hypertensive rats (SHR) have been proposed to have an overactive brain angiotensin system which is critical for their hypertension (Phillips et al. 1988). These rats have an elevated angiotensin receptor density in the brain, particularly in the hypothalamus and brain stem (Saavedra et al. 1992). Central injection of saralasin, an Ang II receptor antagonist decreased blood pressure in the SHR when given centrally at doses that had no effect peripherally (Phillips et al. 1977). Ang II inhibitors did not produce any change in blood pressure of normotensive controls (WKY). These observations suggest that Ang II receptors play an important role in the maintenance of hypertension in SHR. However, the data on losartan (i.c.v.) lowering hypertension in SHR are inconclusive, which may be due to different doses used. The involvement of AT_1 receptor specifically, has been recently revealed by antisense oligodeoxynucleotide (AS-ODN) inhibition of the translation of AT_1 receptor mRNA (Gyurko 1993). AS-ODNs elicit their actions by binding to the mRNA of the specific target protein and inhibiting the protein synthesis. Centrally injected AS-ODN to AT_1 receptor mRNA produced inhibition of high blood pressure in the SHR (Gyurko 1993). Therefore, we hypothesized that AS-ODN to AT_1 receptor mRNA should inhibit effects of direct injections of Ang II. While this report was in preparation, Sakai et al showed that AS-ODN to AT_1 mRNA inhibited drinking to centrally injected Ang II (Sakai et al. 1994). The present study is more extensive as we investigated the effects of AS-ODN on the drinking and vasopressin response to direct i.c.v. injection of Ang II in SHR and Sprague-Dawley rats using the same AS-ODN we had used to reduce hypertension in SHR.

Results

Dipsogenic Response

SHR

Figure 5-1 shows the effect of AS-ODN on the drinking response to Ang II (50 ng) in SHR (n=5) Ang II injection caused an immediate dipsogeness with a water intake of 11 7 ± 0 49 ml/30 min. After pretreatment with AS-ODN, the drinking response to Ang II was significantly reduced to 5 0 ± 0 8 ml/30 min ($P<0.05$). The water intake of the control group which was treated with SC-ODN shows no significant difference with that of animals before ODN treatments

Sprague-Dawley Rats

Figure 5-2 shows the effect of AS-ODN on the drinking response to 50 ng Ang II in normotensive rats (n=5). The water intake induced by Ang II injection was decreased significantly ($P<0.05$) from 6 46 ±1 35 ml/30 min to 2 74 ± 0 95 ml/30 min (after 1 injection of 50 µg of AS-ODN) and to 2.2 ± 0 98 ml/30 min (after 2 injections of 50 µg of AS-ODN) In the repeated test, the second AS-ODN did not further reduce drinking There is no significant difference in water intake after SC-ODN treatments as compared to Ang II alone.

Comparison of Water Intake Elicited by Central Ang II Between SHR and Sprague-Dawley Rats

Figure 5-3 shows the difference in water intake elicited by central Ang II injection in a 30 min period of time between SHR and Sprague-Dawley groups. The water intake for SHR

group (n=5) was 11 7 ± 0 49 ml/30 min which is significantly higher (P<0 05) than that of the Sprague-Dawley group(n=5) which was 6 46 ± 1 35 ml/30 min.

Plasma Vasopressin

Vasopressin was measured in the SHR. The injection of Ang II (50 ng, i.c.v.) increased the plasma AVP from 1.5 ± 0 7 pg/ml to 15 4 ± 0 7 pg/ml (n=5). After 24 hours, a second Ang II injection was administered, and plasma AVP increased to 13 66 ± 0 84 pg/ml. There is no significant difference between the two injections (Fig 5-4). This result showed repeated administration of Ang II in a 24 h interval did not change the level of Ang II induced AVP release and indicated that the protocol of one injection of Ang II followed by a second injection of Ang II was valid.

Figure 5-5 shows the effects of 50 ng Ang II (i.c.v.) injection on AVP release after pretreatment with 50 µg AS-ODN, or SC-ODN or with 4 µl saline control. AS-ODN (n=5) decreased the plasma Ang II induced AVP significantly (6 45 ± 0.54 pg/ml) (P<0 01) compared to the rats pretreated with saline-only (13 04 ± 1.56 pg/ml)(n=5). Compared to SC-ODN treatment group (9 30 ± 0 99 pg/ml)(n=5), the AS-ODN pretreated rats was also significantly lower (p<0 05). However the SC-ODN treatment group had a significantly lower AVP response to Ang II than the saline control group (p<0 05).

Radioligand Binding Assay

Figure 5-6 shows that both total specific binding and AT_1 receptor binding in the hypothalamic block were significantly (P<0 05) decreased in the AS-ODN treatment group

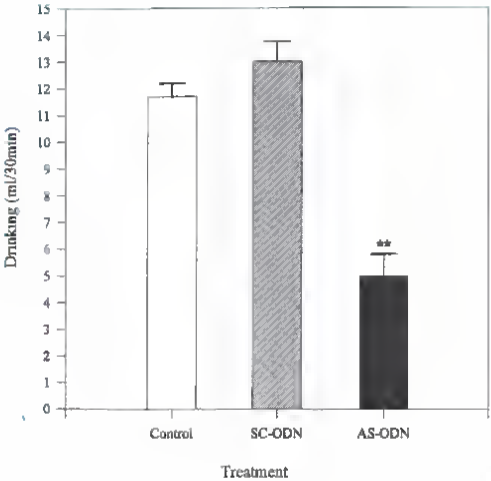

Figure 5-1 Effect of AS-ODN for AT_1 receptor mRNA on drinking to Ang II i.c.v. SHR rats were administered 50 ng Ang II preceded by either saline (unshaded bar), SC-ODN (hatched bar), or AS-ODN (spotted bar). The water intake of each rat in the next 30 minutes was measured. Data are expressed as mean ± SEM (n=5)($p<0.01$)

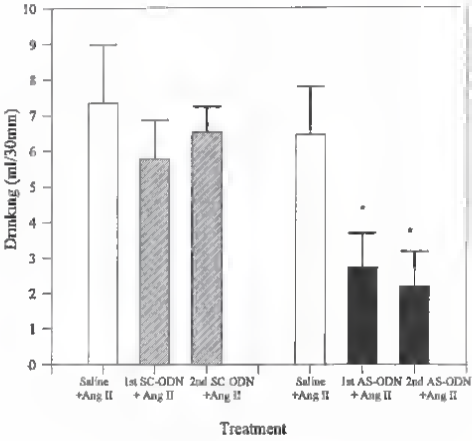

Figure 5-2 Effect of AS-ODN and SC-ODN on drinking with repeated injections SD rats were administered 50 ng Ang II preceded by either saline (unshaded bar), 1 dose of SC-ODN (first hatched bar), 2 doses of SC-ODN (second hatched bar), 1 dose of AS-ODN (first spotted bar) or 2 dose of AS-ODN (second spotted bar) The water intake by each rat in the next 30 minutes was measured. Data are expressed as mean ± SEM (n=5)(p<0 05)

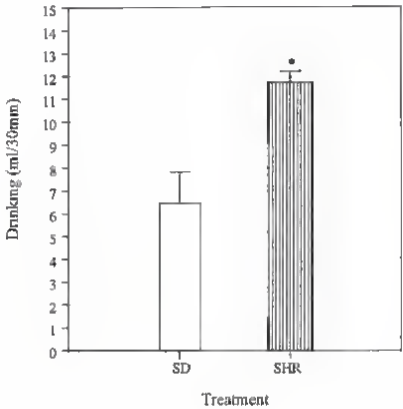

Figure 5-3 The drinking responses of rats to Ang II i.c.v SD (first bar) and SHR (second bar) were administered 50 ng Ang II Water intake of each rat in the next 30 minutes was measured Data are expressed as mean ± SEM (n=5)($p<0.05$)

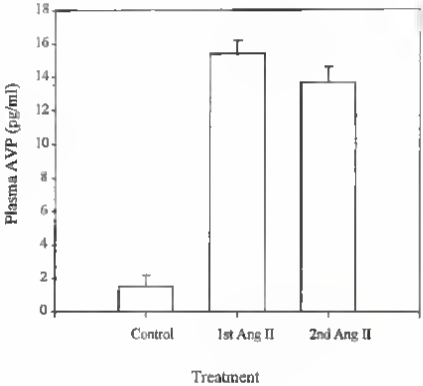

Figure 5-4 Effect of repeated injection of Ang II i.c.v on plasma AVP level Rats were administered either one dose or two doses of Ang II at 24 h intervals. The blood samples were drawn at 1 minute after Ang II injection. The plasma AVP level was measured for each rat Dates are expressed as mean ± SEM (n=5)

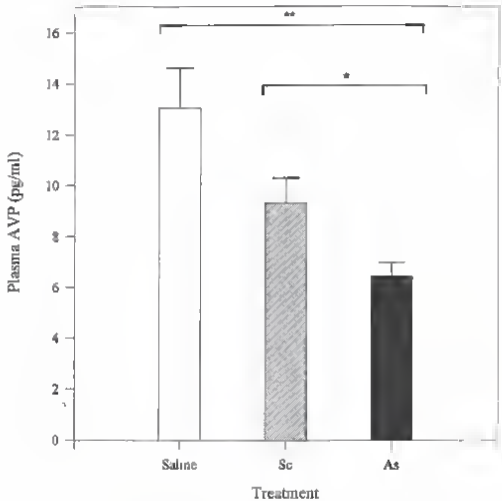

Figure 5-5 Effect of AS-ODN SC-ODN or saline treatment on AVP release to Ang II i.c.v Rats were administered 50 ng Ang II preceded by either saline (unshaded bar), SC-ODN (hatched bar) or AS-ODN (solid bar) The blood samples were drawn at 1 minute after Ang II administration The plasma AVP level was measured for each rat. Data are expressed as mean ± SEM (n=5). ** $p<0.01$ * $p<0.05$

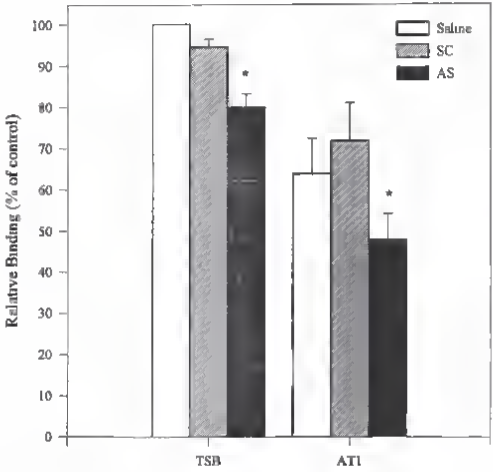

Figure 5-6. Effect of oligodeoxynucleotide treatment on AT_1 receptor binding in the hypothalamic block. TSB = Total Specific Binding AT_1 = Angiotensin II type 1 receptor AS = Antisense Oligodeoxynucleotides SC= Scrambled Oligodeoxynucleotides

compared to the SC-ODN treatment group. The SC-ODN did not change the total specific binding and AT_1 binding significantly from the saline control. Values are expressed as percentage of total specific binding of the saline treated brains.

Discussion

The results show that the AS-ODN to AT_1 receptor mRNA inhibit the physiological effects produced by Ang II (i.c.v.). The data presented here extend the results of Gyurko et al (1993) which showed that central injection of AS-ODN decreased blood pressure in SHR by blocking the protein synthesis of central AT_1 receptor. The results confirm the finding of Sakai et al (1994) that the drinking response to Ang II (i.c.v.) can be inhibited by AS-ODN to AT_1 receptor mRNA, and extend the finding by showing that both drinking and AVP responses to central Ang II were inhibited by AS-ODN in SHR.

Antisense technology was developed as a tool for modulating gene expression. The studies on applying this technology to inhibit gene expression in vivo are just beginning. Wahlestedt et al (1993) injected antisense ODN targeted to NPY Y_1 receptor into the rat cerebral ventricles which resulted in a significant reduction in cortical Y_1 receptor. The report from Ogawa et al (1994) described the antisense ODN targeted to progesterone receptor mRNA lowered the receptor density and inhibited the lordosis behavior. Another successful report comes from studies of Akabayashi et al (1994) in which they inhibited neuropeptide Y synthesis and suppressed feeding behavior by using the antisense ODN to neuropeptide Y mRNA. In the present study AS-ODN suppressed dipsogenesis and AVP release induced by

Ang II injected into brain. The working hypothesis of AS-ODN action is that AS-ODN inhibit AT_1 receptor expression by interfering with translation of AT_1 receptor mRNA (Helene et al. 1990 and Akhtar et al. 1992). Confirming this mode of action, we found decreased AT_1 receptor density in the hypothalamic region after AS-ODN treatment.

Interestingly the AS-ODN did not completely inhibit the drinking response. Even with a repeated dose of AS-ODN treatment the inhibition was not significantly greater than with a single injection. When comparing the percentage decrease in drinking with the SC-ODN treated group as the control, we found that the decrease in drinking with the first dose of AS-ODN was 52.6% and with the second dose of AS-ODN the decrease was 66.3%. This result is consistent with the report of Hogarty et al. (1992) which showed that central injection of losartan, an AT_1 receptor antagonist reduced but did not completely block the dipsogenic response induced by Ang II (i.c.v.). This may suggest that other non-AT_1 receptors are involved in mediation of the drinking response. Hogarty et al. proposed that perhaps AT_2 receptors were involved. It is also possible that other receptors not yet identified may also play a role in central Ang II induced drinking. Although non-AT_1 receptor mediated dipsogenesis is an attractive idea, the possibility of the AT_1 receptor accounting for all of the drinking response still exists. The autoradiographic analysis of Ang II receptor sites in the hypothalamus region indicated that AS-ODN treatment resulted in a maximum of 40% decrease in the SHR (Gyurko et al. 1994). This suggests that a higher dose of AS-ODN or repeated AS-ODN treatment should be able to block more AT_1 receptors thus completely inhibiting Ang II dipsogenesis. However in our receptor binding study we used three injections of AS-ODN (50 µg/injection). With this regimen only a 40% decease in AT_1 receptor density in

hypothalamic region was seen in the Sprague-Dawley rats. This is consistent with our in-vivo studies where we showed that a second AS-ODN injection did not further decrease drinking beyond the first injection. The lack of increased effects with repeated administration of AS-ODN indicates that antisense does not completely inhibit the receptor protein synthesis. This may be due to limited uptake in critical cells or the AT_1 receptor gene responds by upregulating AT_1 receptor synthesis. It seems unlikely that the feedback mechanism would be fast enough or efficient enough to compensate for repeated doses of AS-ODN. If the rate of ODN uptake is the rate-limiting step, even with repeated administrations, the cells may not take up any more AS-ODN. The excess AS-ODN may be diluted in CSF and then degraded. Despite the profound decrease in dipsogenic response, the observed decrease in AT_1 receptor binding was smaller. This may be a result of substantial decreases in AT_1 in discrete nuclei being masked by other areas with greater AT_1 receptor density in the dissected tissue block. Therefore it is conceivable that a relatively small change in receptor number causes dramatic decrease in the physiological response. An alternative explanation is that the life cycle of AT_1 receptor is giving a false picture. The life cycle involves internalization and recycling. During these stages the receptor is not active but detectable by the receptor binding assay. This would mask the true decrease in binding of active membrane-bound receptors by the antisense. The SC-ODN had no effect on drinking response, which showed that the action of the AS-ODN is sequence-specific.

In the response to central Ang II, SHR drank 80% more than SD rats. These data add support to the hypothesis that SHR has overactive RAS components in the brain compared to the normotensive Sprague-Dawley rats. Sakai et al also reported that injection of AT_1 receptor

AS-ODN into the third ventricle partially inhibited the drinking response to Ang II in normotensive rats (Sakai et al 1994) They also showed that AS-ODN had no effect on central carbachol induced drinking. Their study obviated the need to include the carbachol induced drinking, but we have addressed the question about Ang II inhibition in SHR and effects on AVP release

The main target sources of AVP release by Ang II are SON and PVN of the hypothalamus which have high AT_1 receptor density Yang et al (1992) showed that Ang II depolarization of SON neurons was inhibited by losartan and Hogarty et al (1994) showed that losartan could inhibit Ang II induced AVP release. The AS-ODN treatment significantly decreased the AVP release induced by central injection of Ang II, providing further support for AS-ODN inhibition of AT_1 receptor expression The SC-ODN control also slightly decreased AVP release, although the effect of AS-ODN was significantly greater The inhibition of AVP release after SC-ODN treatment compared to saline controls is an empirical finding for which we have no explanation. In the experiment on drinking, SC-ODN had no inhibitory effect on the drinking response to central Ang II AVP release is related to osmolarity changes and studies suggest that AVP response to a rise in plasma osmolality is mediated by, or involves at some point, an angiotensinergic pathway in the brain (Hogarty et al 1994, Sladek et al. 1980 Yamaguchi et al. 1982) The sensitivity of Ang II induced AVP release to AS-ODN for AT_1 mRNA inhibition supports this view AVP release is one of the postulated mechanisms by which SHR maintain hypertension The present results are consistent with this concept and may be relevant to the decrease in hypertension in SHR with AS-ODN

The present research is in its early stage. Fine tuning of the modification of AS-ODN and improved delivery methods will ultimately allow smaller doses to be delivered to sites of action as well as enhancing or optimizing the cellular uptake efficiency. This ultimately would allow the administration of smaller doses of AS-ODN with equal or greater potency. Overall this would have fewer potential side-effects unlike some of the other chemical receptor antagonists. This is advantageous for both development as a therapeutic agent and a physiological tool. In addition, a further advantageous is the modified AS-ODN has been shown to elicit effects for extended periods of time, unlike receptor antagonists which have effects that are relatively short acting. In summary, this report demonstrates a new approach to modulate the AT_1 receptor gene expression in the rat brain. Our results confirm the function of the AT_1 receptor in controlling drinking and AVP release and also provide a potential new tool to regulate the physiological effects mediated by the AT_1 receptor in the brain.

CHAPTER 6
CHARICTERIZATION OF DENDRIMER BASED GENE DELIVERY SYSTEM *IN VIVO* AND ITS APPLICATION IN TREATING RESTENOSIS

Introduction

Restenosis is the process of reobstruction of an artery following interventional procedures such as angioplasty atherectomy, or stenting. This disease is a multifactorial process. The migration and proliferation of VSMCs is most likely to the most consequential event (Clowes et al. 1988) Conventional drug therapeutic approaches have focused on either preventing platelet deposition, thrombus formation or inhibiting VSMCs growth. However, the problems with drug administration have prevented conventional drug therapy from achieving any clinical significance (Hermans et al 1993) Therefore, many researchers have turned their attentions to a new approach - Gene therapy Gene therapy is one of the fastest growing fields in the biomedical research. This emerging branch of medicine aims to correct genetic defects by transferring genetic materials into cells. One of the most dynamic research areas is antisense oligonucleotide (AS-ODN) based gene inhibition AS-ODNs are considered a new class of therapeutic drugs that consummate their functions by binding to mRNA in a sequence-specific manner Traditionally drugs work on the protein levels. Although they can inhibit protein functions, they usually need repeat administration. Non-specific effects and protein upregulation associated with drug inhibition are frequently observed Antisense technology was introduced to overcome these shortcomings of traditional drugs Many successful reports have

indicated that antisense approach is a very useful tool in manipulating gene products Because of the fast metabolic degradation for natural occurring phosphodiester oligonucleotide, but the phosphorothioate prolongs the activity of antisense ODNs. At present, there are two ways to deliver AS-ODN, direct and incorporated into a delivery system. Direct administration is limited by low cellular uptake and fast elimination *in vivo* of AS-ODNs Liposomes formulations, such as cationic, have the potential to enhance cellular uptake of polynucleotides into mammalian cells. However, the low efficiency and cyto-toxicity of liposomes have limited this approach *in vivo*

Starburst dendrimers® are a new class of macromolecules first described by Dr Donald A. Tomalia in the 1980s (Tomalia et al 1985) These polyamino spherical molecules have highly branched, tree-like structures terminating in a surface of primary amines having the ability to bind anionic nucleic acids Dendrimers are classified by the number of cascade polymer generations required As the generation number increases there is a corresponding increased in number of primary amines and molecular weight. A newly-emerging area in dendrimer technology is the delivery of genetic material into the cell Many *in vitro* reports have ascertained dendrimers are able to deliver genetic material efficiently into many cell types without damage to the organisms (Boussif et al 1995, Haensler et al 1993) The delivery of AS-ODN by dendrimers as vector is being studied by other groups (Schwab et al 1994, Poxon et al 1996) They have been reported to have many advantages over other liposome and other particulate based delivery systems The advantages of their products include defined polymerization reaction, reproducible product low toxicity and the ability to alter the transport and binding characteristics by changing the generation of dendrimer used However, the *in*

vivo data about toxicity and metabolism need to be completely studied before dendrimers can be developed into drug delivery systems.

In the present study, we have used polyamidoamine Starburst® dendrimers which were synthesized from an ethylene diamine core, resulting in a series of primary amine groups on the outermost sphere. We investigated the pharmacokinetics and tissue uptake of generation 4,6 and 10 fluorescent labeled dendrimers, free fluorescent labeled oligonucleotides, and generation 6 DEN electrostatically complexed to fluorescent labeled ODN.

In present study, we also explored the potentiality of using dendrimer based delivery system to delivery AS-ODN to treat restenosis. The biggest problem associated with using antisense strategy to fight restenosis is achieving sufficient cellular uptake of the oligo and maintaining the antisense inhibition long enough to inhibit neointimal growth. Therefore, a suitable delivery system for AS-ODN is needed. Local delivery rather than systemic administration is a more effective way to obtain higher tissue drug levels at the site of the balloon injury. Local delivery can also minimize the potential side effects. Several local drug delivery systems, including perfusion balloon catheters, hydrogel-coated balloon catheters, polymeric or coated stents, and many other approaches are currently under investigation. However, the low tissue uptake of the agents remains the main disadvantage of the catheter injection systems (Fernandez-Ortiz et al. 1994, Mitchel et al. 1995, Lincoff et al. 1994, Fram et al. 1994). Blood flow washes out the agents in minutes to hours. We have investigated the dendrimer as a sustained-release carrier system that could enter the vascular wall rapidly and not be washed out. We have also tested the ability of using dendrimer complexed with AS-

ODN to AT_1 receptor mRNA to decrease neointimal formation after vascular injury in the same animal model

Results

Purification of Dendrimers

Dendrimers were labeled by a simple conjugation using fluorescein isothiocyanate and the primary amines from the dendrimer (Poxon et al 1996) After the reaction the unreacted fluorescein needed to be isolated from the dendrimers Spin columns filled with G-10 Sephadex were used for the purification of FITC-labeled DEN from unreacted label Samples were run through spin columns for three times until there was no detectable free FITC signal on TLC plates Figure 6-1 illustrates the purity of the dendrimer labeling reaction by TLC purification procedure for the 4th generation DEN The signals of free FITC label indicated by lane 1 were gradually decreased until it could no longer be detected after the third time spin This purification step guaranteed us for using pure FITC-labeled DEN for the rest of experiments Similar results were obtained for the other dendrimer generations

Dendrimer:Oligonucleotide Reaction

In order to determine the interaction of oligonucleotides and dendrimers we performed gel retardation experiments (Fig 6-2) In this method the anionic oligonucleotides easily migrate through the gel matrix towards the cathode As the net charge of the complex is changed due to the addition of the cationic dendrimers at first the

movement of the complex is slowed but with the addition of increasing amounts of dendrimer the moment stops and is finally reversed towards the anode Using this method we have calculated that 1 mole of dendrimer could react with 8 moles of oligonucleotide resulted in complete binding of the oligonucleotide. In essence the study demonstrated dendrimers could complexed with oligonucleotide and at some ratio form a positively charged complex

Pharmacokinetics

The determination of pharmacokinetical parameters with particulate delivery systems is often a difficult process because it requires having the ability to quantitate both the drug of interest and the delivery vector In this set of experiments we could take advantage of easy labeling methods to attach fluoresecent reporters (fluorescein isothiocyanate) to both the delivery vector (DEN) and the ODN By varying the administration of the ODN and DEN we could generate data for the ODN free DEN and the complex. Table 6-1 demonstrates the pharmarcokinetical parameters of different samples that we tested These values indicated that as the dendrimer generation increases there is a corresponding increase in half life of elimination. Also from the data presented the ODN-DEN complex appears to be stable in the blood stream since there was a corresponding increase in the elimination half-life (Fig 6-3) For example, the 6th generation of PAMAM DEN and found that it significantly ($p<0.01$) increased the elimination half time of 15 mer oligo ($t1/2\alpha$ and $t1/2\beta$) from (3.45 ± 0.58 39.55 ± 5.97) to (9.00 ± 3.35, 395 ± 53) min (Fig 6-4)

Tissue Distribution of DEN

Tissue distribution suggested that 24 hours after infusion, FITC-Den were accumulated in kidney, liver and blood vessels (Fig 6-5) They did not cross the blood brain barrier into brain and there was not a significant amount of signal detected skeletal muscles

Effect on Neointimal Formation

DEN-ODN (100 μg) were delivered in situ to rat left common carotid artery Treatment with the complexes of AS-ODN and dendrimer significantly reduced neointimal formation compared to the control The treatments with AS-ODN alone and with SC-ODN complexed to dendrimer yielded no significantly changes (Fig 6-6)

TABLE 6-1 PHARMARCOKINETICAL PARAMETERS OF DENDRIMERS

Generation	$t_{1/2\alpha}$ (min)	A_1	k_1	$t_{1/2\beta}$ (min)	A_2	k_2
4th	1 68	48 5	0 41	20 05	71 0	0 035
6th	4 07	70 61	0 17	51 67	32 88	0 0134
10th	2 93	58 8	0 236	83 66	30 9	0 008
15merODN	3 45	18 65	0 201	39 55	8 54	0 0175
CF	2 8	43 5	0 255	19 76	43 55	0 035

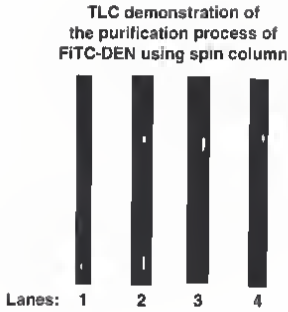

Fig 6-1 Purification of FITC labeled Dendrimers (4th Generation) Dendrimers were labeled by a simple conjugation using fluorescein isothiocyanate and the primary amines from the dendrimer Samples were run through spin columns filled with G-10 Sephadex three times until there was no detectable free FITC signal on TLC plates The signals of free FITC label indicated by lane 1 were gradually decreased until it could no longer be detected after the third time spin (lane 4) Lane 1, Free FITC Lane 2, 1st time spin Lane 3 2nd time spin Lane 4, 3th time spin Similar results were obtained for the other dendrimer generations

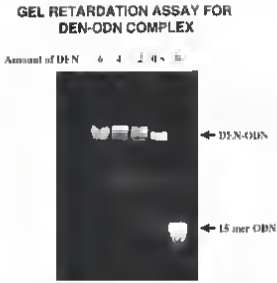

Fig 6-2 Gel retardation experiment on ODN-DEN complex The anionic oligonucleotides migrate through the gel matrix towards the cathode As the net charge of the complex is changed due to the addition of the cationic dendrimers at first the movement of the complex is slowed but with the addition of increasing amounts of dendrimer the moment stops and is finally reversed towards the anode.

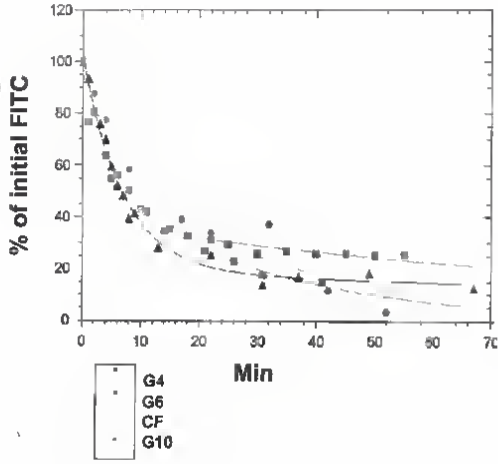

Fig 6-3 Serum elimination of generation 4, 6 and 10 dendrimer and CF

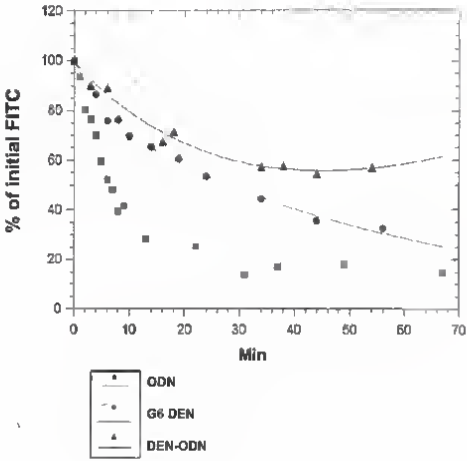

Fig 6-4 Serum elimination of generation 6 dendrimer Free 15 mer ODN and DEN-ODN complexes

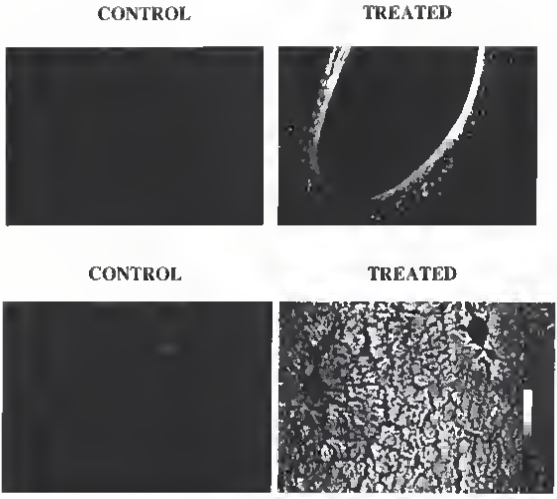

Fig 6-5 Tissue distribution of Generation 6 dendrimer Twenty four hours after infusion, FITC-Den were detected in liver and blood vessels Left panel, Control Right panel Treated

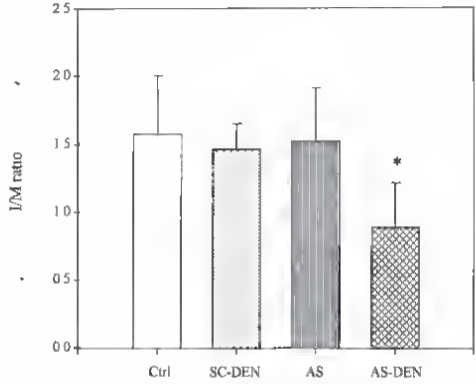

Fig 6-6 Effect of AS-ODN for AT_1 receptor on neointimal formation Balloon injured rats were treated with control (n=5) SC-DEN (n=4) AS alone (n=4) and AS-DEN (n=4) The AS-DEN treated rats showed significant decrease on the ratio of intimal/medial areas

Discussion

Various attempts to alter the restenosis process by conventional pharmacological or mechanical approaches (eg stenting) have not yet proven complete success in a clinical setting. The antisense oligonucleotide approach could open a new avenue for the treatment of this disease. Many reports have shown that antisense strategy holds a lot of potential. However, there has to be a suitable delivery system for antisense therapy. There are normally two ways to administer drugs in restenosis research, systemically and locally. Conventional drugs are administered per os to be effective systemically such as ACE inhibitor and AT_1 antagonists. There are some possibilities to administer AS-ODNs systemically, or even per os especially to use modified ODNs and carrier system to protect ODNs from degradation. In present study, we addressed this problem by injection dendrimer/ODN complex into rats and achieved significant uptake into blood vessels. However, it is generally acknowledged among restenosis researchers that local delivery might be the most efficient approach. Local drug delivery facilitates high regional concentrations of therapeutic agents with prolonged retention. Local drug delivery reduces systemic toxicity by lowering circulating doses. In addition, therapeutic agents with short half-life, such ODNs can be delivery locally with minimal loss of therapeutic activity. Sine the balloon catheterization that leads to restenosis is done in surgery the application of AS-ODN at this time is appropriate. Current local delivery methods include adventitial drug implant, stents, catheter infusion etc. Adventitial delivery have been successful in experimental restenosis. Simons et al used a pluronic gel to delivery AS-

ODN to *c-myb* and inhibited restenosis in rats (Simons et al. 1992) Coating stents is a potentially useful technique for achieving sustained local administration Hydrogel catheters and porous catheters are also promising systems Although these delivery systems can enhance the uptake and local concentration of ODNs that they deliver (Shi et al 1994) they will not be able to increase the stability of ODNs

Cationic liposomes are the prevailingly gene delivery system in antisense research Cationic lipids often with dioleoylphosphatidylethanolamine (DOPE) as an additional lipid component, form positively charged liposomes This feature allows binding of oligonucleotides which are negatively charged The main mechanism of the cellular delivery is thought to be endocytosis (Lappalainen et al 1994) Cationic lipids have been used to delivery AS-ODNs to cell culture and enhance uptake of ODN Zelphati et al (1996) show that addition of the liposome/ODN complexes to cultured cells results in a dramatic increase in nuclear accumulation of the fluorescent ODN The inhibitory activity of antisense ODN is also increase by liposome which may suggest that nuclear localization on ODN correlates with an enhanced inhibitory activity However there are several drawbacks which potentially prevent cationic lipids from developing as therapeutic agents Cationic liposomes are normally serum sensitive. The transfection of cells needs be carried out in serum free media Thus serum effect may preclude the use cationic liposomes for any intravascular ODN delivery (Felgner et al 1987) Cytotoxicity is another drawback for this delivery system It often make it difficult to determine specific antisense effects in vivo (Wagner et al 1993)

Nanoparticles which share some features of dendrimer is another promising delivery system for AS-ODN therapy in restenosis. Nanoparticles are 30-500 nm diameter polymeric spherical particles. There are several different types of biodegradable polymers available including biopolymers (gelatin, albumin, casein, polycyanoacralate lectins, etc.) and synthetic polymers (polycyanoacralate, polyesters, polyanhydride, polycaprolactione, etc.). They have various drug release characteristics ranging from several hours to several months. Oligonucleotides adsorbed onto nanoparticles have been showed to have enhanced stability against nucleases and with a more ideal cellular deposition (Chavany et al. 1992, Godard et al. 1995). Chavany et al. (1994) have demonstrated an increase in half life of nonoparticle-bound oligonucleotide from 2 min to over 1200 min when exposed to snake venom phosphodistrase. Wilensky et al. (1995) demonstrated that arteries were able to retain radioactive latex microparticles delivery using standard porous balloon catheters in experimental animal for up to 7 days.

Through the use of these particulate systems investigators have been able to alter the pharmacokinetics profile of the entrapped ODN. Unfortunately, the particulate nature of the delivery system is a limitation. We have been working on PAMA dendrimers as an alternative delivery system for gene therapy since 1995. Dendrimers are produced through a cascade polymerization resulting nanoparticles with ending branch with a polyamino surface. These molecules range in size from 10 to 100 nm, with each generation of the polymer adding ~10 nm to the diameter of the molecule. The number of surface primary amino groups doubles with each generation, reaching 4960 for tenth generation of dendrimer (Tomalia 1995). At physiological pH (7.4) the majority of the

amino groups are ionized. Thus there is a cationic surface which can interact with anionic molecules electrostatically. The defined structure of these molecules and their large number of surface amino groups has led to dendrimers being employed as a substrate for the attachment of any bioactive molecules which are negatively charged. PAMAMA Dendrimers have been shown to complex with antibodies. Studies using antibody-dendrimer complex in experimental animals have documented these complexes to be non-toxic and cells specific. Dendenmers have also demonstrated the ability to deliver oligonucleotides (Bielinska et al. 1996), and plasmid DNA (Kukowska-Latallo et al. 1996) to a variety of cultured cells. In these cases a new molecular identity is formed between the opposite charged molecules. At most ratios of dendrimer complexed to oligonucleotides the complex is soluble and not prone to aggregation. The new formed complex has been shown to be resistant to nuclease degradation and to enhance cellular uptake of ODN in tissue. Stability of oligonucleotides is a necessary requirement for the application of antisense technology to inhibit gene expression in vivo. This often precludes the use of naked phosphodiester ODNs because of the fast degradation in serum. Significant efforts have been made toward the development of nuclease-resistant oligonucleotides in particular phosporothioates and methylphosphosntaes. However, these modification may increase toxicity and non-antisense effects. Dendrimers provide us with the potentiality of using phosodiester ODNs which are the natural forms of DNA. Bielinska et al (1996) showed that dendrimers could enhance both stability of uptake of phosphodiester ODNs. This may eventually allows us to use the natural form of ODNs in place of expensive and sometimes toxic modified ones. Interestingly the binding of ODNs

to dendrimer does not interfere with the physiological effects of ODNs It is possible that the binding of the DDN phosphate backbone does not change the property of bases to form hydrogen bonds with the complementary sequences.

Dendrimer have the ability to achieve prolonged systemic circulation effect Conjugation of DDN to dendrimer increased the elimination half of ODN in blood Our result show that the pharmacokinetics data best fit into a two compartment model in which contain α and β elimination phases The (t1/2α and t1/2β) were generation 4 (1 68 20 05), generation 6 (4 07 51 67) and generation 10 (2 9, 83 66) min respectively We evaluated the 6th generation of PAMAM DEN for the electrostatic interaction with ODN and found that it significantly ($p<0.01$) increased the elimination half time (t1/2α and t1/2β) from (3 45 ± 0 58, 39 55 ± 5 97) to (9 00 ± 3 35, 395 ± 53) min. The similar increase in elimination are also reported on modified nanoparticle/ODN complexes (Tan et al 1993) The complexes of ODN and dendrimer result in the increased size of the particles. One molecule of generation 6 dendrimer was shown to be able to absorb 8-10 molecules of ODN in our study The significant increase in the size of complexes may be responsible for the prolonged elimination half time

We started the delivery of AS-ODN with pluronic gel Pluronic gel is able to deliver phosphorothioated AS-ODN into VSMC when they were apply on adventitia together Using FITC labeled DDN and confocal microscopy we showed that AS-ODN was able to diffuse into media and maintained there for up to 48 hrs However, AS-ODN for AT_1 dissolved in 20% pluronic gel did not cause any significant reduction in restenosis in our lab This may be attributed to the fast degradation and short tissue retention of DDN in

blood vessels After we applied the ODN/dendrimer complexes, we found that AS-ODN began to function and achieved ~50% reduction in neointimal formation We conclude that the dendrimer delivery is capable of increasing stability, uptake and efficiency of AS-ODN

There has been no report on toxicity of dendrimers In our study, we have not observed any toxic effects so far However there is always some concerns Since the dendrimer may not be made from biodegradable materials, repetitive administration may eventually lead to accumulation and toxicity More study still need to be carried out to answer this question For the present study it was only necessary to give the DEN AS-ODN once so toxic effects of repeated doses are not an issue

In summary our study demonstrate that dendrimers are highly efficient delivery system for ODN therapy They facilitate uptake of ODN into tissues and enhance the stability and inhibitory effect of AS-ODN

CHAPTER 7
GENERAL CONCLUSIONS

The components of RAS have been located in vascular system in human, rats and other animals. Vasoconstrictor Ang II has been shown to promote cellular hypertrophy and hyperplasia in many cell types including VSMCs. The growth functions of Ang II have been given important consequences regarding restenosis induced by angioplasty. However, previous studies which were based on ACE inhibitors and AT_1 antagonists failed to achieve uniform conclusion in this issue. The resource of the controversies is the different doses and time of dosing. Other problem such as receptor upregulation may also contribute to the failure of these drugs. This research project used two novel techniques, autoimmunization and antisense inhibition to address this problem.

We first tested the effectiveness of the AS-ODN to the AT_1 mRNA on a well characterized animal model in our laboratory, centrally Ang II induced drinking and AVP release. Given 50 μg of AS-ODN to the lateral ventricle of rats significantly inhibited drinking and AVP response to Ang II. Our results indicate that antisense strategy can be successfully used in RAS. We exploited dendrimer based delivery system to facilitate uptake of AS-ODN into blood vessels. Dendrimer significantly increased uptake and stability of AS-ODN. AS-ODN to AT_1 mRNA delivered with dendrimer significantly inhibited neointimal formation after balloon injury.

The other strategy we used was autoimmunization against AT_1 receptor protein. We immunized rats with a peptide corresponding to the N-terminal of AT_1 receptor. The immunized rats and control rats then were subjected to balloon catheterization. Our results demonstrated that immunized rats had significant less neointimal formation compared to sham control. Further, we transfused antiserum into normal rats and inhibited neointimal growth. We conclude that when Ang II and the AT_1 receptor were inhibited by an autoantibody to the N-terminal of the AT_1, the growth response to the vascular injury was significantly inhibited.

In summary (Fig. 7-1), our data with autoimmunity to the AT_1 receptor confirm and extend the hypothesis that vascular RAS is important for the mechanism involved in the development of restenosis. Our study suggests that Ang II is one of the initiating factors in the response to vascular injury. The present study also demonstrates that AS-ODN is effective in reducing restenosis and we have developed a dendrimer delivery system for administering AS-ODN efficiently for prolonged effects. We conclude that AS-ODN complex dendrimer delivery offers a potentially new therapeutic approach for restenosis and vascular response to injury.

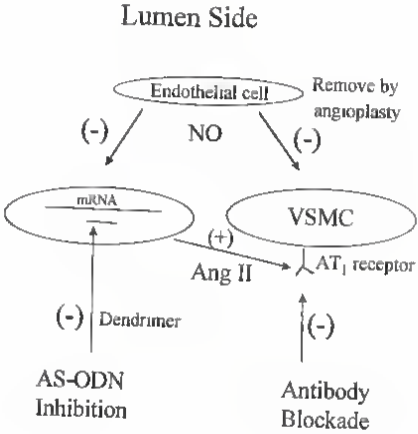

Fig. 7-1 Summarization of physiological events following angioplasty and inhibitory functions of autoantibody and antisense on the AT_1 receptor

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BIOGRAPHICAL SKETCH

Frank Meng was born in Beijing, 1968. His parents are both professors. He found biomedical research to be intriguing to him from a young age. He received numerous awards and recognition during his high school years in a variety of competitions in the biomedical field. This experience laid the foundation of his decision to become a researcher in this interesting field. Frank attended Beijing Medical University from 1986 to 1991, and received his Bachelor of Science degree. To seek new challenges and higher education, he moved to Gainesville and enrolled in the Ph.D program in medical sciences with a specialization in physiology at University of Florida in 1992. Frank is married to Jane and they have a lovely family. During free time, he enjoys tennis, biking, swimming, and traveling.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy

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Stephen Baker
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Colin Sumners
Professor of Physiology

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Bruce Stevens
Professor of Physiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality as a dissertation for the degree of Doctor of Philosophy

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This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy

December, 1997

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